

ASSESSMENT OF CHROMATIN ACTIVITY IN MOUSE AND HUMAN TISSUES

by

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ABSTRACT

Pancreatic deoxyribonuclease preferentially digests active genes during all phases of the cell cycle including mitosis. Recently, a DNase I-directed in situ nick translation technique has been used to demonstrate differences in the DNase I sensitivity of euchromatic and heterochromatic regions of mitotic chromosomes. This in situ technique has been used in this study to ask whether facultative heterochromatin of the inactive X chromosome can be distinguished from the active X chromosome in mouse and human tissues. In addition to this, in situ nick translation has been used to distinguish constitutive heterochromatin in mouse and human mitotic chromosomes. Based on relative levels of DNase I sensitivity, the inactive X chromosome could not be distinguished from the active X chromosome in either mouse or human tissues but regions of constitutive heterochromatin could be distinguished by their relative DNase I insensitivity. The use of in situ nick translation was also applied to tissue sections of 7.5 day mouse embryos to ask whether differing levels of DNase I sensitivity could be detected between different tissue types. Differences in DNase I sensitivities were detected in three tissues examined; embryonic ectoderm, an embryo-derived tissue, and two extraembryonic tissues, extraembryonic ectoderm and ectoplacental cone. Embryonic ectoderm and extraembryonic ectoderm nuclei possessed comparable levels of DNase I sensitivity while ectoplacental cone was significantly less DNase I sensitive. This suggests that tissue-specific mechanisms such as chromatin structure may be involved in the regulation of gene activity in certain tissue types. This may also shed some light on possible tissue specific mechanisms regulating X chromosome activity in the developing mouse embryo.

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INTRODUCTION

Pancreatic deoxyribonuclease (DNase I) preferentially digests genes that are or have been transcriptionally active (Weintraub and Groudine, 1976). This digestion has been attributed to an altered, more accessible configuration of the surrounding chromatin. Inactive chromatin is characterized by a more closed configuration that protects the DNA from nuclease attack. This DNase I sensitive conformation is maintained in fixed mitotic chromosomes, thus, DNase I sensitivity serves as a useful structural probe for gene expression (Gazit et al., 1982).

Recently, the use of DNase I-directed techniques have demonstrated differences in the DNase I sensitivities of euchromatic and heterochromatic regions of mitotic chromosomes (Kerem et al., 1983, 1984; Murer-Orlando and Peterson, 1985; Kuo and Plunkett, 1985; Adolph and Hameister, 1985). Kerem et al., (1983) reported that in situ nick translation could distinguish the active and inactive X chromosome of the gerbil on the basis of differing DNase I sensitivities. The use of such an assay to detect the inactive X chromosome would be of valuable assistance in studying the process of X-inactivation in the developing mouse embryo. In the mouse embryo, X-inactivation is a unique developmentally regulated event that is thought to occur concomitantly with specific differentiative changes during embryogenesis. X-inactivation in the embryo is characterized by a unique inactivation pattern that is generally random in embryonic cell lineages but nonrandom in extraembryonic cell lineages due to the preferential inactivation of the paternally derived X chromosome. How and why this nonrandom pattern of X chromosome inactivation occurs and is restricted to extraembryonic cell tissues is still not clear. X-inactivation can take place in at least three distinctive stages of development; preimplantation,

early implantation and mid-gestation, but the actual timing or onset of X-inactivation and the specific progenitor cells in which it first occurs is also not known. The availability of a technique that distinguishes the inactive X chromosome on the basis of differing DNase I sensitivity would suggest that structural differences exist between the chromatin of the active and inactive X chromosome. This would implicate chromatin structure as a possible mechanism regulating X chromosome activity. One could then ask whether structural differences exist between the X chromosomes from embryonic and extraembryonic tissues of the mouse embryo and whether the chromatin structure of the inactive X chromosome is the same in both tissue types. The use of such a technique would be useful in providing information about possible tissue specific mechanisms regulating X chromosome activity in different cell lineages of the developing mouse embryo.

There has been some controversy in the literature as to whether in situ nick translation actually results in the identification of the inactive X chromosome. Several studies have reported no observable differences in the DNase I sensitivity of the active and inactive X chromosome or sequences from the X chromosome using in vitro or in situ DNase I-dependent nick translation techniques (Riley et al., 1984; Murer-Orlando and Peterson, 1985; Adolph and Hameister, 1985). Therefore, it was of primary interest to carry out a detailed study to determine whether in situ nick translation could distinguish differences in the DNase I sensitivity of active and inactive X chromosomes from mouse and human tissues. In addition to this, there was also some controversy reported in the literature as to whether in situ nick translation could identify regions of transcriptionally inactive constitutive heterochromatin

of mitotic chromosomes. Constitutive heterochromatin shares many properties with facultative heterochromatin except that its chromatin structure is more condensed and it remains condensed and transcriptionally inactive throughout the cell cycle. The second aim of this study was to ask whether in situ nick translation could distinguish constitutive heterochromatin on the basis of differing DNase I sensitivity.

The final aim of this study was to develop a standard DNase I treatment that would allow one to distinguish overall differences in the DNase I sensitivity of cells from different tissue types. This would be particularly useful if applied to different cell lineages of the developing mouse embryo. Tissue specific modifications of DNA have been reported in a number of studies and have been implicated as possible underlying mechanisms controlling transcriptional activity. Differences have been found to exist between embryonic and extraembryonic cell lineages of 7.5 day embryos with respect to levels of DNA methylation of repetitive DNA sequences (Chapman et al., 1984). Modifications by way of methylation were found to be reduced in extraembryonic cell lineages compared to embryonic cell lineages. DNA from the inactive X chromosome was found to be modified in adult and embryo-derived tissues but not in extraembryonic tissues (Liskay and Evans, 1980; Kratzer et al., 1983). The development of a DNase I-sensitive technique would allow one to examine possible tissue specific differences in chromatin structure based upon differing DNase I sensitivities. This would perhaps result in a greater understanding of possible mechanisms regulating tissue specific modifications of X chromosome DNA and consequently of activity. This involved using in situ nick translation and 7.5 day embryo tissue sections so that the DNase I sensitivity of cells from different tissue types could be assessed in situ.

In summary then, using DNase I-dependent techniques, the questions that were addressed in this study are as follows:

1. Can facultative heterochromatin of the inactive X chromosome be distinguished from the active X chromosome by in situ nick translation?
2. Can constitutive heterochromatin be distinguished in mitotic chromosomes by the same technique?
3. Can overall differences in DNase I sensitivity be detected between cells of different tissues by in situ techniques?

LITERATURE REVIEW

X-INACTIVATION IN THE DEVELOPING MOUSE EMBRYO

In the mammalian system, females possess two X chromosomes and males possess an X and a Y chromosome. The Lyon hypothesis states that one X chromosome becomes inactivated in each somatic cell of the early female embryo while the single X chromosome of XY male somatic cells remains active (Lyon, 1972). This pattern of inactivation ensures that the dosage relationship between the sex chromosomes and autosomes of the female will be the same as in the somatic tissues of the male. X-inactivation also prevents gene imbalance in males or females with more than their normal number of X chromosomes. Males that are XXY or females that are XXX will normally have all but one X chromosome inactivated in their somatic tissues. Other characteristics attributed to the inactive X chromosome include a generally more condensed chromatin structure that is visible as condensed sex chromatin or Barr body in interphase nuclei (Barr and Bertram, 1949) and a differential or heteropycnotic staining pattern in mitotic chromosomes (Tagaki and Oshimura, 1973). In addition to this, the inactivated X chromosome is late replicating, beginning and ending replication during S phase later than the other chromosomes of the complement (Tagaki, 1974). In adult somatic tissues, the inactive X chromosome can be of paternal (X^p) or maternal (X^m) origin. Once the decision is made as to which chromosome will be inactive, all descendants of that cell will have the same parental chromosome inactivated. Thus in adult somatic cells, X chromosome inactivation is said to be random but

fixed, resulting in one inactive X chromosome in each female somatic cell. Several hypotheses have been proposed to explain the phenomenon of X-inactivation such as DNA modification by way of methylation, the binding of regulatory substances to the X chromosome and the concept of "inactivation centres" on the X chromosome (Kratzer et al., 1983; Mohandas et al., 1981; Lyon, 1972; Rastan, 1983). The possible involvement of conformational changes in chromatin structure as an underlying mechanism regulating X-inactivation also provides interesting speculation (Kerem et al., 1983).

X-inactivation in the developing mouse embryo is a developmentally regulated event that occurs early in embryonic life. X-inactivation in the mouse embryo does not show the same pattern as seen in adult somatic cells. Instead, regional and temporal differences are observed in the inactivation pattern affecting the appearance, randomness of inactivation and the pattern of DNA replication of the inactive X-chromosome. X chromosome inactivation occurs concomitantly with specific differentiative changes during embryogenesis and does not occur simultaneously in all cells of the embryo. Examination of various cell lineages revealed that the pattern of X-inactivation is not always random. The extraembryonic cell lineages, derived from trophoctoderm and primary endoderm, demonstrate a non-random inactivation pattern whereby the paternally derived X chromosome is preferentially inactivated (Takagi and Sasaki, 1975; West et al., 1977; Harper and Monk, 1983). Replication studies of the very early embryo also revealed that the ^PX chromosome replicated earlier during S phase than the autosomes or the active _mX chromosome (Takagi et al., 1982). The

^P
 inactivated X chromosome became late replicating in extraembryonic lineages only later in development. These observations can be classified as events occurring in at least three distinctive stages of development; preimplantation, early implantation and mid-gestation, but the actual timing or onset of X-inactivation and the specific progenitor cells in which these phenomena occur is still not clear.

The earliest sign of X-inactivation was found to occur concomitantly with the formation of trophoctoderm during the blastocyst stage at 3 days of development. The second incident of X-inactivation takes place at 4.5 days of development, concomitantly with the formation of primary endoderm (Monk and Harper, 1979). In both cases X-inactivation is limited to those cells that have undergone differentiation. In those cells remaining pluripotent, two X chromosomes continue to function. By 6.5 days of embryonic development, X-inactivation has occurred in the majority of the cells although some cells, particularly germ line progenitor cells in the embryonic ectoderm, still possess two active X chromosomes (McBurney and Strutt, 1980). By 11.5 days of development, X-inactivation has occurred in the germ cells that have entered the genital ridge and developed in oogonia (Kratzer and Chapman, 1981). The sum effect of this pattern of inactivation is that one X chromosome has been inactivated in every cell of the developing embryo.

Preimplantation Embryo

1. Zygote - Late Blastocyst Stage:

The status of the X chromosome in the zygote is unknown but it is

assumed that only the maternally derived X chromosome is active. The paternally derived X chromosome is thought to be in an inactive state since it is inactivated during spermatogenesis (Lyon, 1972). Indirect evidence leading to the assumption that the maternally derived X chromosomes were active during oogenesis was initially made by Epstein (1969). Using an X-linked enzyme, glucose-6-phosphate dehydrogenase (G6PD) and an autosomally-linked enzyme, lactate dehydrogenase (LDH), enzyme activity distributions of Mus musculus oocytes were quantitated. G6PD and LDH are two enzymes that are abundantly produced in mouse ova (Brinster, 1965; 1966). To monitor X chromosome activity, the oocytes were derived from female mice either of XO or XX constitution. This would allow one to predict a 2-fold difference in the levels of G6PD activity of XO- and XX-derived oocytes and no differences in LDH activity. Results showed that G6PD activity of XX oocytes was twice that of XO oocytes and the LDH activity was the same in both. This suggested that both X chromosomes are active in mouse oocytes and that the maternally derived X chromosome passes in an active state into the zygote. Similar evidence was also presented in a later study by Epstein, (1972) using hypoxanthine guanine ribosyl transferase (HGPRT) activity analysis, another X-linked enzyme. Again HGPRT activity levels were found to be proportional to the number of X chromosomes present in each oocyte.

More direct evidence was made available by the discovery of electrophoretic variants of X-linked enzymes HGPRT, G6PD and PGK (phosphoglycerate kinase) (Chapman et al., 1983; Nielsen and Chapman, 1977). Conveniently detectable electrophoretic variant alleles have been

used in several studies demonstrating that both X chromosomes are active in mouse and human oocytes up to the time of ovulation (Kratzer and Chapman, 1981; Migeon and Jelalian, 1977). In the study reported by Kratzer and Chapman, Mus caroli G6PD-A females were crossed with G6PD males of the same species. X chromosome expression was determined by G6PD heterodimer A/B activity in G6PD A/B oocytes, indicating that both X chromosomes were active. X chromosome activity during oogenesis was also investigated using PGK-1 expression and an X-autosome translocation, Searle's translocation (T(X;16)16H), in mouse embryo germ cells (Johnston, 1981). Since PGK-1 is a monomer, electrophoretic analysis would not distinguish variant allelic expression within or between cells. The Searle's translocation can then be utilized in an indirect method of assessing X chromosome activity since the X-autosome translocation chromosome would preferentially be kept active. Mice heterozygous for PGK-1 alleles and Searle's translocation would then only express the alleles located on the active translocation chromosome if X-inactivation had occurred. Moreover, both X chromosomes were found to be functional in oocytes, confirming the results of Kratzer and Chapman, (1981). In summary, the maternally derived X chromosome is considered to be active in the zygote while the paternally derived X chromosome is not.

Assessing X chromosome activity in the early embryo, Epstein (1972) reported the expression of both X chromosomes in day 1 (2-cell) embryos and day 3 (morula) embryos using an X-linked enzyme. This activity was also detected in individual embryos of the 8 - 16 cell stage and morula stage using activity distributions of X-linked HGPRT (Kratzer and Gartler, 1978). HGPRT is an embryonically transcribed enzyme that is synthesized

during preimplantation development in the mouse embryo. Before chromosome inactivation, female embryos with two X chromosomes would be expected to show twice as much enzyme activity for an X-linked enzyme than male embryos with only one X chromosome. This would be visualized as a bimodal enzyme activity distribution with two peaks separated by a factor of two. Should X-inactivation have taken place, a unimodal distribution would then be predicted due to dosage compensation. Additional evidence was presented by Epstein et al. (1978) and Kratzer and Gartler (1981) using HGPRT activity of preimplantation embryos ranging from the 2 cell stage up to the very late blastocyst stage of development. Both X chromosomes were found to be active at the 8 cell stage and very early blastocyst stage (Kratzer and Gartler, 1981). In the late blastocyst stage there was a shift in the enzyme activity ratio between females and males from 2:1 to 1.5:1. By the very late blastocyst stage of development, the activity distribution was unimodal and the ratio between female and male embryos was 1:1. This suggested that X-inactivation had occurred at some point between the morula stage and the blastocyst stage, coincident with the first differentiation process to occur in the developing embryo, that of trophectoderm formation. Interpretation of the HGPRT activity data of early and late blastocysts presented by Monk and Kathuria (1977) suggested that X-inactivation was seemingly limited to the trophectoderm since it constituted the majority of the cells found in the blastocyst. Gardner and Lyon (1971) were able to show that those cells of the pluripotent inner cell mass (ICM), still possessed two functional X chromosomes. Single cell injection into a mouse blastocyst producing a chimera was used as a cell clone marker system to

study X-inactivation in the developing embryo. X chromosome activity was monitored using coat color. If the female donor cell possessed as active X chromosome, white coat patches would be visible in the chimeric individual. If X-inactivation occurred before cell transfer, coat color would consist of dark wild-type patches and sandy-colored patches only. The production of chimeric mice with all three coat colors suggested that X chromosome inactivation had not occurred in the single donor cell. However, the production of some chimeric mice with only wild-type and sandy coat patches again indicated that X-inactivation began at 3.5 days of blastocyst development or shortly after.

Combined biochemical and cytological evidence indicating that two X chromosomes are functional in preimplantation embryos was also presented by Epstein et al., (1978). HGPRT enzyme activity distributions of early and late, male and female blastocysts were assayed. The previously described enzyme assay studies monitoring X-chromosome activity assumed that the lower enzyme activity peaks were produced by male embryos expressing a single X chromosome. In this study, the sex of the embryos was determined by karyotyping one of the "twin half-blastocysts" obtained by separating blastomeres of the 1.5 day, 2-cell embryo and culturing them in parallel. The male to female ratio of HGPRT enzyme activity was found to be approximately 2:1 in very early half-blastocysts and 1:1 in late blastocysts (12-18 hours after blastulation). This again indicated that X-inactivation was concomitant with the first differentiative event that occurs with blastocyst formation.

Cytological evidence supporting the occurrence of X-inactivation in

day 3 embryos was presented by Mukherjee (1976) and Tagaki (1974). Inactivated X chromosomes generally demonstrate differential staining properties (heteropycnosis), allocyclic DNA replication patterns and sex chromatin formation (Lyon, 1972). Tagaki (1974) used quinacrine mustard fluorescence and acetic saline Giemsa staining to delineate the allocyclic X chromosome in preimplantation embryos. Female mouse embryo cells in the early cleavage stage were found to be isopycnotic, in that both X chromosomes showed the same characteristic banding patterns. At the 40-50 cell stage, one of the X chromosomes became heteropycnotic and by the 90-cell blastocyst stage, delineation of the inactive X chromosome was quite evident. Changes in the DNA replication pattern of the inactive X chromosome were also studied by Mukherjee (1976) using cell cycle analysis. It was found that up until the blastocyst stage, early embryos lack a G_1 phase in their cell cycle. Mukherjee contended that the appearance of the G_1 period during blastocyst stage of development coincides with the altered replication pattern of one of the X chromosomes that has been inactivated. Thus, the biochemical and cytological results collectively suggest that the first occurrence of X-inactivation seems to coincide with the first differentiative event that takes place in the embryo.

2. Implantation

X-inactivation also occurs coincident with a second differentiative event that occurs in the developing embryo, the formation of primary endoderm (Monk and Harper, 1979). Monk and Harper specifically looked at X-chromosome activity in extraembryonic ectoderm (trophectoderm derived),

primary endoderm (ICM derived) and embryonic ectoderm using HGPRT enzyme activity distribution analysis. In 6 day old embryos, the activity distributions shown in extraembryonic ectoderm and primary endoderm were unimodal, suggesting that only one X chromosome was active in female embryos. The embryonic ectoderm activity distribution was still bimodal, indicating two active X chromosomes. However, the results for embryonic ectoderm were not absolute. The bimodal enzyme distribution suggested that X-inactivation in the primitive ectoderm had occurred in approximately one-fifth of the female embryos. By 6.5 days of development, HGPRT enzyme activity distributions were unimodal, revealing that X chromosome inactivation had occurred in the majority of cells. Although the exact timing of the inactivation event in primary ectoderm is still not defined it was assumed that it occurs at approximately 6 days of gestation. These findings collectively indicated that there exists a regional and temporal pattern of X chromosome inactivation that occurs concomitantly with specific differentiative changes in the developing mouse embryo.

Preferential Inactivation of X^P

Not only was there an observed regionalization of the embryo with respect to appearance of the inactive X chromosome and its replication pattern but there were also regionalized differences in the parental origin of the chromosome inactivated. The mosaic constitution of the extraembryonic cell lineages proved to be quite different from the embryonic lineages. In extraembryonic tissues, preferential inactivation of the paternally derived X chromosome was evident, resulting in a biased

mosaic constitution (Tagaki & Sasaki, 1975; West et al., 1977; Tagaki et al., 1978; Tagaki et al., 1982). Embryonic cell lineages were characterized by the "random but fixed" X-inactivation pattern normally found in adult somatic tissues. Much of the evidence for this is cytological, often involving X-autosome translocation chromosomes as cytological markers. Tagaki and Sasaki (1975) used a cytologically distinguishable X-autosome translocation marker, Cattanach's translocation, to distinguish X^m and X^p in 6.5 and 8.5 day embryos. Inactivity was detected using the differential staining pattern of the inactive X chromosome (Takagi and Oshimura, 1973). The inactive X was found to be of paternal origin in approximately 50% of the mitotic figures examined from embryo derived cells while 90 % of the extraembryonic derived cells contained an inactivated X^p . This preferential pattern of X inactivation in extraembryonic cell lineages was confirmed by additional studies done by Tagaki et al (1978; 1982). The nonrandom inactivation pattern was detected as early as the blastocyst stage of development (Tagaki et al., 1978). Using Cattanach's translocation, T(X;7)1Ct, to cytologically detect the inactivated X chromosome, the X chromosome was found to be heteropycnotic in 87-88 % of the blastocyst cells examined.

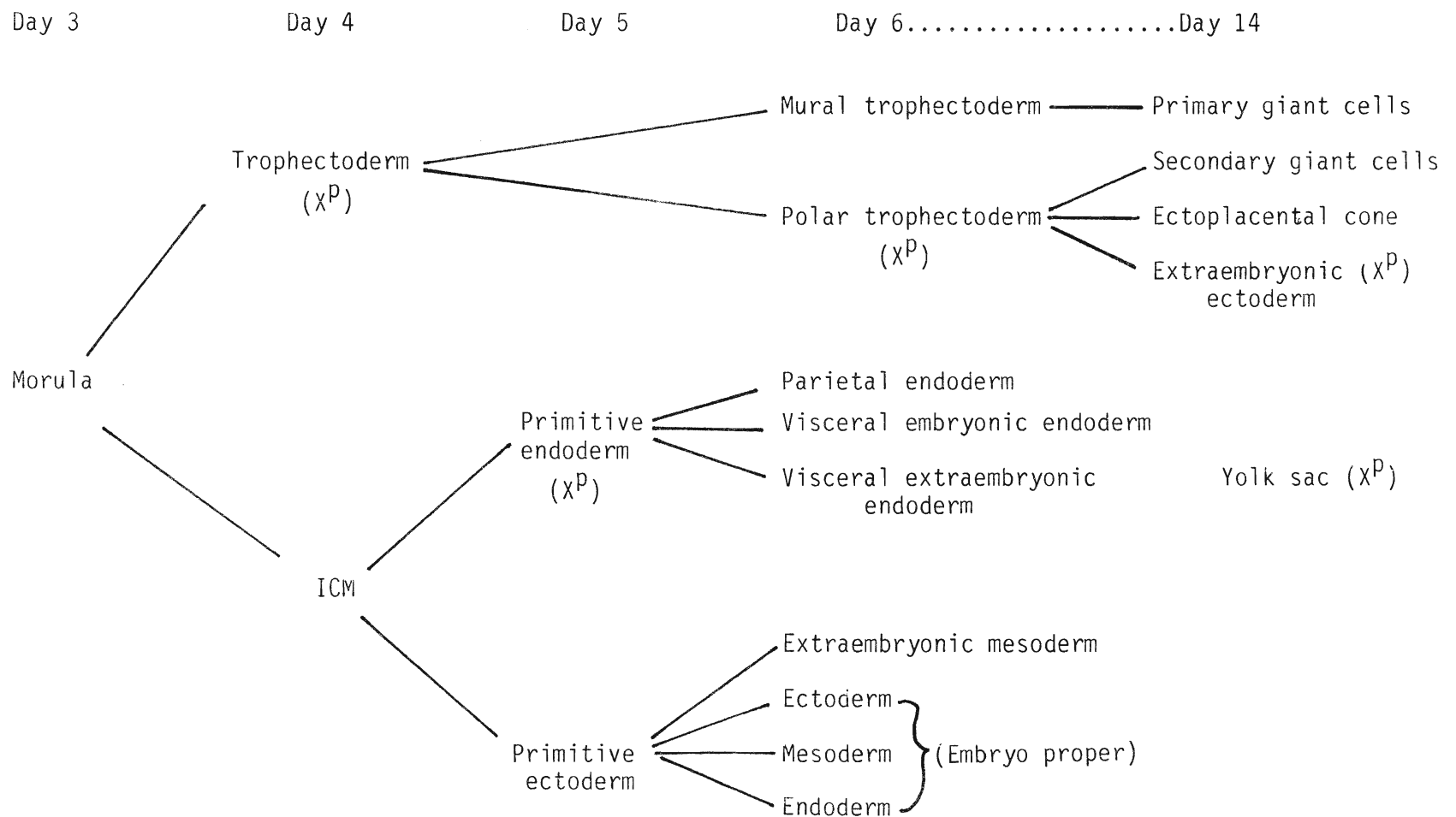
Biochemical evidence demonstrating preferential expression of X^m in an extraembryonic cell lineages was reported by West et al. (1977), essentially confirming the results reported by Tagaki and Sasaki (1975). Using X-linked electrophoretic variants of PGK-1, the parental origin of the inactive X chromosome was estimated by the relative proportion of X^p and X^m expression in 14 day mouse embryo and yolk sac tissue. Yolk sac, an

extraembryonic cell lineage, preferentially expressed the maternally derived allele while both the maternal and paternal alleles were expressed in equal proportions in the embryo. The paternally derived PGK-1 allele was not thought to be expressed in yolk sac endoderm.

A detailed study of the X chromosome inactivation pattern in the developing mouse embryo was conducted by Tagaki et al. (1982). Regionalization of the embryo in terms of the temporal appearance, parental origin and DNA replication pattern of the allocyclic X chromosome was observed. The results showed that in embryonic tissues, one X chromosome became late replicating at approximately 6 days of development and inactivation was random with respect to parental origin. However, in the extraembryonic tissues, the allocyclic X chromosome appeared before 5.3 days of development and in the majority of cases was paternally derived. The pattern of X^P replication in the extraembryonic tissues was unique in that it was early replicating instead of late as in embryonic tissues. In certain instances a transitory pattern was observed in the replication pattern; in extraembryonic ectoderm, 79.4 % of the cells had an early replicating X^P at 5.3 days of development. By 6.5 days of development, 74.9 % of the X^P chromosomes were late replicating. This regional switch in the replication pattern of the allocyclic X is not characteristic of all extraembryonic cell lineages, only extraembryonic ectoderm. These data clearly show the overall preferential inactivation of the paternally derived X^P in extraembryonic tissues.

The cytological and biochemical data presented collectively suggest that X-inactivation occurs concomitantly with the first two differentiative

Figure 1. Schematic diagram of embryonic and extraembryonic lineages of mouse conceptuses (Kratzer et al., 1983). Cell lineages containing an inactivated paternally derived X chromosomes are designated X^P.



events to occur during embryonic development in the mouse and that X^P is not expressed in derivatives of trophectoderm nor in tissues derived from primary endoderm. No preferential inactivation pattern of X^P occurs in cell lineages derived from ICM. A summary of the stages of development and tissues in which X inactivation has occurred is presented in Figure 1.

POSSIBLE MECHANISMS REGULATING X-INACTIVATION

A number of hypotheses have been presented to try to explain the maintenance of X chromosome differentiation in mammals. DNA modification by way of methylation has attracted a lot of attention but results are often conflicting. Chromatin structure and DNase I sensitivity, non-histone proteins and polyanionic substances have also been proposed as possible regulators of genetic activity. Martin (1982) hypothesized the existence of a marker influencing the preferential inactivation of the X^P chromosome that is tissue specific.

DNA Modification of the Inactive X chromosome

Evidence suggesting that DNA modification of the inactive X chromosome is prevalent in adult mouse somatic tissues was initially presented by Liskay and Evans (1980) using DNA mediated gene transfer. The rationale for using this approach is based upon the assumption that differential modification of DNA would result in inefficient transformation efficiencies. Should X-inactivation be the result of differential modification of DNA alone and not due to regulating chromosomal proteins or other factors not directly affecting the DNA, then one would predict that

DNA mediated gene transfer would demonstrate inefficient transfer of an allele on the X chromosome. Alternatively, if X-inactivation is not the result of DNA modification, then alleles on the active and inactive X chromosome would have equal transformation efficiencies. Electrophoretic variants of the X-linked HGPRT enzyme were used as a marker system to detect active and inactive HGPRT alleles. The DNA was isolated from a HGPRT⁻ mutant CAK mouse cell line that has the active X chromosome carrying a HGPRT mutation and the inactive X carrying the wild-type HGPRT allele. The HGPRT on the inactive X chromosome did not transform the HGPRT Chinese hamster cell line. As a control, DNA isolated from a wild-type HGPRT⁺ CAK cell line did result in the transformation of the HGPRT⁻ cell line. This suggested that the DNA of the HGPRT gene on the inactive X chromosome was different from the DNA coding for the same gene on the active X chromosome, indicating that the DNA is modified at or near the HGPRT gene on either X chromosome. This conclusion, however, was based upon the assumption that the HGPRT gene was intact on the inactive X chromosome of the CAK cell line.

Chapman et al (1982) provided direct in vivo evidence that DNA modification was involved in the maintenance of X chromosome inactivation in adult mouse tissues. Again electrophoretic variants of HGPRT were used along with an X-autosome translocation marker T(X;16)16H. Adult female mice, heterozygous for T(X;16)16H, only express X-linked genes found on the translocation chromosome in their somatic tissues since the normal X chromosome is preferentially inactivated. Consequently, females also heterozygous for HGPRT electrophoretic variants consistently express the

HGPRT allele found on the active translocation chromosome. DNA was isolated from heterozygous T(X;16)16H females and its transformation efficiency was assayed using DNA-mediated gene transfer. The transformation abilities of the active and inactive X chromosome were determined by the HGPRT electrophoretic variant type expressed by the transformant lines. Unlike the active X chromosome DNA, DNA from the inactive X chromosome of a number of adult tissues was not efficient in HGPRT transformations. This provided direct in vivo evidence that the DNA of active and inactive X-linked mouse HGPRT sequences were differentially modified without assuming that the HGPRT gene was intact on the inactive X chromosome.

Tissue specific differences in the transforming abilities of inactive X chromosome DNA were also shown to exist between embryonic and extraembryonic cell lineages in the 14 day mouse embryo (Kratzer et al., 1983). Crosses of mice were obtained so the female embryos produced were heterozygous for HGPRT and PGK-1 alleles. By this means, expression of maternal and paternal alleles could be detected electrophoretically. Transformation efficiencies of X-chromosomes from yolk sac endoderm, yolk sac mesoderm and adult liver were assessed by DNA mediated gene transfer of the HGPRT gene. Liver and yolk sac mesoderm are derivatives of primitive ectoderm and show random inactivation of either the X^m or X^p chromosome. Yolk sac endoderm is derived from primitive endoderm and shows preferential inactivation of X^p . DNA isolated from HGPRT a/b yolk sac mesoderm and adult liver transformed cell lines so that either the maternal or the paternal alleles were expressed. When DNA isolated from 14 day yolk sac

endoderm was used in the gene transfer assay, the transformant cell lines again expressed either the maternal or paternal HGPRT allele. Since the X^P chromosome was preferentially inactivated in this tissue, transformants from both X^m and X^P indicated that both the active and inactive X chromosome were equally efficient in gene transfer. This suggested that the DNA of the inactive X chromosome in yolk sac endoderm was not differentially modified from that of the active X chromosome. This suggests that even though a single X chromosome is inactivated in both embryonic- and extraembryonic-derived cells containing two X chromosomes, tissue-specific differences in DNA modification of X chromosome DNA do exist. DNA of the inactive X chromosome from embryonic tissues was modified while DNA from the inactive X chromosome from extraembryonic tissues was not.

DNA Modification By Methylation

A mechanism for modification that could account for the inactivated state of the X chromosome is that of DNA methylation. Methylation of mammalian DNA is found in the ubiquitous form of 5-methyl-cytosine (Razin and Riggs, 1980). Methylation of DNA involves enzymatically converting cytosine bases to 5-methyl-cytosine (m cyt) by methyl transferase (Razin and Riggs, 1980). The methyl group is transferred from 5-adenosyl methionine to position 5 of cytosine. Depending on the species, 2-7% of the total genomic cytosine is converted to m cyt (Vanyushin et al., 1970). There are a number of possible ways in which methylation of cytosine could influence or regulate the biological function of DNA (Felsenfeld et al.,

1982). Methylation could alter the structure of DNA and consequently that of chromatin by causing the DNA to interact differently with histones, non-histone proteins or regulatory proteins. Whatever the means of alteration, levels of DNA methylation are often correlated with changes in chromatin structure.

Methylated cytosine residues can be detected by a variety of restriction enzymes with the isoschizomer restriction endonucleases Hpa II and MspI being the most commonly used. Both restriction enzymes recognize the same sequence, CCGG, but Hpa II will not cleave the sequence C CCG^m if the internal cytosine residue is methylated. Msp I is able to cleave both methylated and unmethylated CCGG sequences. A commonly used technique that detects methylated cytosine residues was initially introduced by Bird and Southern (1978). This technique usually involved cutting total genomic DNA with Hpa II or Msp I, separating out the DNA fragments by agarose gel electrophoresis and then blotting them out onto a nitrocellulose sheet. DNA fragments in or near the gene of interest can then be hybridized with labelled RNA or cloned DNA probes and visualized by autoradiography. Levels of DNA methylation could then be assessed by the prevalence of DNA fragments produced when restricted with Hpa II.

DNA Methylation and Gene Activity

An inverse correlation is said to exist between levels of DNA methylation and gene activity. Because of this association, methylation has been implicated as part of a secondary mechanism regulating gene expression (Doerfler et al., 1982). DNA methylation was first suggested as

a possible regulatory control when tissue-specific differences in levels of methylation were observed in the rabbit (Waalwijk and Flavell, 1978). Rabbit DNA was restricted with Msp I or Hpa II, electrophoresed on an agarose gel and analysed using Southern blot hybridization with a labelled β -globin specific probe. The restriction fragments were found to be methylated in a tissue specific manner at a site within the β -globin gene intron. The cytosine residues of DNA isolated from rabbit brain, sperm and liver were hypermethylated compared to the cytosine residues of DNA isolated from a rabbit cell line.

The first correlation of gene expression and site-specific DNA methylation was presented by McGhee and Ginder (1979) using chicken β -globin genes. Msp I and Hpa II restriction analysis of cells that expressed the β -globin gene (adult reticulocytes and erythrocytes) appeared to be undermethylated at CCGG sites near the end of the gene sequence. These same sites appeared to be at least partially methylated in cells that did not express the β -globin gene.

Tissue specific methylation patterns were more recently reported in several distinct cell lineages of the mouse embryo by Chapman et al. (1984). Using two cloned Mus musculus middle repetitive sequence probes, DNA isolated from different embryonic and extraembryonic early cell lineages was assessed for levels of DNA methylation using Msp I and Hpa II restriction analysis. In an earlier study done Saloman et al. (1969), mouse satellite DNA was found to be highly methylated, almost twice that of main band DNA. Chapman et al. found that the repetitive DNA from two extraembryonic cell lineages, trophoctoderm and primitive endoderm, were

significantly undermethylated compared to primitive ectoderm derivatives (derived from the embryo proper) and adult somatic tissues.

Extraembryonic tissues, extraembryonic ectoderm and ectoplacental cone, and embryonic tissues of 7.5 day mouse embryos were also assessed, in this same study, for differing levels of DNA methylation using a minor satellite sequence probe. Again, extraembryonic tissues were found to be substantially undermethylated compared to embryonic and adult liver tissue. These results suggested that aspects of gene regulation dependent upon DNA modification may differ in embryonic and extraembryonic tissues of the developing mouse embryo.

DNA Methylation and X-inactivation:

The differences detected in the transformation efficiencies of alleles on the active and inactive X chromosome initially suggested that the DNA of the inactive X chromosome was differentially modified in adult and embryo derived tissues. Methylation has been more directly implicated in the process of X-inactivation by a variety of means. Hypomethylation of DNA can be induced by 5-azacytidine (5-azaC), an analogue of cytidine, which when incorporated into DNA causes impaired DNA methylation (Jones and Taylor, 1980). Mohandas et al (1981) reactivated genes on the inactive X chromosome in mouse-human somatic cell hybrids using 5-azaC, thereby providing evidence for the involvement of methylation in X-inactivation. The mouse-human somatic cell hybrid clone was deficient for HGPRT activity and contained an inactive human X chromosome. Reactivation of the inactive X chromosome 5-azaC was determined by the expression of human X-linked

genes, HPRT, G6PD and PGK in 14 clones. HPRT was expressed in all clones and G6PD was expressed in one clone and PGK in another.

Wolf and Migeon (1982) on the other hand, did not observe any differences in levels of DNA methylation of the active and inactive X chromosome DNA nor did 5-azaC treatment result in the derepression of the inactive X chromosome in clonal populations of normal human fibroblasts. Using two cloned DNA fragments unique to the X chromosome, levels of DNA methylation in placentas, and male and female skin fibroblasts were assessed. The use of the cloned DNA fragment provided a more direct way of looking at differences in levels of methylation between the active and inactive X chromosome DNA. Levels of DNA methylation were detected using Msp I and Hpa II restriction analysis as previously described for Waalwijk and Flavell (1978). No consistent differences in DNA methylation were observed with respect to X chromosome activity in normal human cells. There were some site specific differences observed between cloned male and female cell lines, depending on what X-DNA probe was used and some variability was observed in the methylation of a single Hpa II site in uncloned male and female fibroblast cell lines. The variability, however, was not consistent and this difference was attributed to differences in methylation from cell to cell. Treatment of normal female skin fibroblasts with 5-azaC did not result in the re-expression of G6PD and HGPRT alleles in the inactive X chromosome. Methylation of human X chromosomes in mouse human hybrid cells was also assessed. The predominance of a single restriction enzyme band in all the cell hybrids was suggested by Wolf and Migeon to be the result of a more stable methylation pattern found in a foreign

environment. From these results, Wolf and Migeon concluded that X DNA methylation was not significantly correlated with X chromosome transcriptional activity in normal human fibroblast cells.

In more recent studies, however, it was found that the HPRT gene does exhibit differing levels of DNA methylation on the active and inactive X chromosome (Mohandas et al., 1981; Wolf et al., 1984; Yen et al., 1984). The gene was found to be undermethylated on the active X chromosome but differently methylated, albeit at highly variable levels, on the inactive X chromosome. The differing levels of methylation were mainly localized to sequences 5' to the HPRT gene. From these results, it can best be said that DNA methylation is not the primary mechanism regulating overall X chromosome activity but may be involved in the regulation of specific genes on the X chromosome. Additional evidence supporting this is provided by the observation that X-inactivation occurs in extraembryonic tissues of the mouse embryo in the absence of DNA modification differences. Extraembryonic tissues are generally characterized by lower levels of DNA methylation than embryonic tissues, suggesting that X-inactivation is regulated by some other mechanism. Since DNA methylation is not strongly implicated as a primary mechanism regulating X chromosome activity, conformational changes in chromatin structure may be involved as a possible means of regulatory control.

CHROMATIN STRUCTURE AND GENE ACTIVITY

DNase I Sensitivity as a Structural Probe for Gene Expression

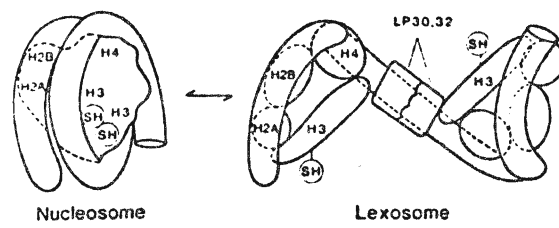
Pancreatic deoxyribonuclease is one of the most widely used nonspecific endonucleases in chromatin studies. A useful indicator of chromatin activity, DNase I recognises internal features of nucleosomes and preferentially degrades active or potentially active genes on the basis of altered nucleosome structure (Reeves, 1984). This DNase I hypersensitivity has been attributed to an altered, more accessible 'open' configuration of the chromatin surrounding the active gene. DNase I appears to recognize at least three different aspects of active chromatin structure; (1) regions of "open" chromatin surrounding specific, potentially active genes, (2) regions of chromatin characterized by an intermediate level of DNase I sensitivity and (3) DNase I hypersensitive sites generally located at the 5' or 3' ends of coding regions (Reeves, 1984). All three aspects of DNase I sensitivity, thought to reflect regulatory changes in chromatin structure, have been implicated in both tissue specific levels of DNase I hypersensitivity and developmental control of gene expression.

Hierarchy of Chromatin Structure

1. Nucleosome

The nucleosome is the first level of chromatin organization thought to occur in the nucleus. It consists of a core particle containing an octamer of histones H4₂, H3₂, H2A₂, H2B₂ and approximately 146 basepairs (bp) of double B form DNA wrapped around it in two left-handed superhelical forms

Figure 2. A model illustrating the nucleosome-lexosome transition in ribosomal genes of Physarum as proposed by Prior et al. (1983). The compact nucleosome particle extends into the open form of the lexosome involved in the transcription of ribosomal genes. (Reprinted from Prior et al., 1983).



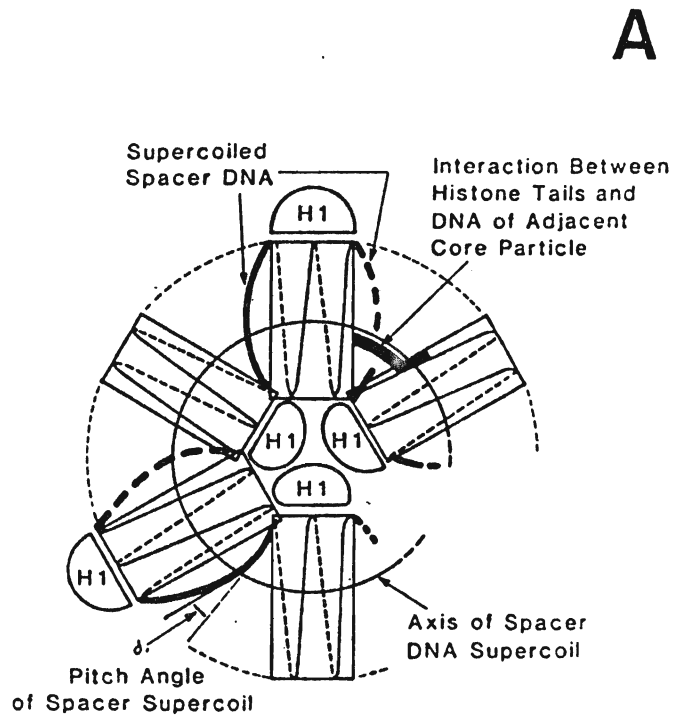
(Reeves, 1984; Igo-Kemenes et al., 1982; Weisbrod, 1982; Mathis et al., 1980). Confirmed by X-ray diffraction analysis (Finch et al., 1981), neutron scattering (Finch et al., 1980), chemical cross-linkage studies (Mirzabekov et al., 1978) and electron microscope image reconstruction techniques (Klug et al., 1980), the nucleosome particle is a flattened, wedge-shaped disc that is approximately 11 nm in diameter and 5.7 nm in height (cited in Reeves, 1984). Nucleosomes are arranged on a 10 nm chromatin fiber in a repeating array with intermittent linker or spacer DNA (0 - 80 bp length). In addition to nucleosomes, chromatosomes, defined as nucleosomes with an additional 20 bp of linker DNA and one histone H1 molecule on each side of the nucleosome structure, are also alternatively spaced on the 10 nm fiber. The DNA packing ratio, defined as the ratio of extended DNA length to its unit length in the fibre, is of the order of 6-9 in a 10 nm fiber (Suau et al., 1979, cited in Reeves, 1984).

The structures of nucleosomes and chromatosomes are not static in nature but rather undergo structural and compositional changes (Reeves, 1984). Prior et al. (1983) have demonstrated that active regions of Physarum rDNA chromatin are in an extended form compared to inactive rDNA chromatin. Electron microscopy analysis has shown that the majority of the extended subunits consist of two sphere-like bodies connected by a 50 bp nucleoprotein bridge, referred to as the extended lexosome (L) form. A model proposed by Prior et al. hypothesizing the nucleosome-lexosome transition is shown in Figure 2.

2. Solenoids

The next level of chromatin compaction is in the form of a shallow

Figure 3. Cross section (A) and sideview (B) of 30 nm solenoid model proposed by McGhee and Felsenfeld (1980). The solenoid configuration consists of chromatosomes radially arranged with approximately 6 nucleosomes per turn, creating a chromatin fibre equivalent to that of bulk chromatin.



supercoiled solenoid. This is postulated to be the result of winding or coiling of the 10 nm fiber into the structure shown in Figure 3. The solenoid configuration, proposed by McGhee and Felsenfeld (1980), consists of chromatosomes radially arranged with approximately 6 nucleosomes per solenoid turn. This creates a chromatin fiber of approximately 25 - 30 nm, representing the bulk of chromatin that exists in vivo (Olins and Olins, 1979). The packing ratio of the 30 nm fiber is in the range of 40 - 50:1, similar to that of interphase chromatin (Finch and Klug, 1976). As with nucleosomes, solenoids undergo continual transitions from the 10 nm fiber to the 30 nm fiber. Histones H1 and H5 have both been implicated in stabilizing the higher order of coiling of chicken erythrocyte chromatin with H1 being less tightly bound (Allan et al., 1981). One would predict that removal of histone H1 would result in the unwinding or unfolding of the solenoid structure. Selective unfolding of chicken erythroid chromatin in the region of the active β -globin gene has been demonstrated by Kumura et al. (1983), and a number of studies have shown that active chromatin regions appear to be depleted of histone H1 proteins compared to inactive bulk chromatin (Itzaki, 1971; Berkowitz and Doty, 1975; Weintraub and Groudine, 1976; Burkholder and Weaver, 1977). It has also been debated that certain non-histone proteins of the high mobility group (HMG's) replace the histone H1 molecules in active regions of chromatin. Active fractions of sonicated reticulocyte chromatin contained twice as much non-histone protein and 15% less histone than the inactive fractions (Berkowitz and Doty, 1975). Burkholder and Weaver (1977) observed that chromatin contained both histone and non-histone proteins but that

non-histone proteins were more tightly associated with the DNA in condensed chromatin than in extended chromatin. This would suggest that non-histone proteins could be responsible for the differential DNase I sensitivity between extended and condensed chromatin.

3. Chromatin matrix

The third level of chromatin compaction found in interphase nuclei and metaphase chromosomes is the result of folding of the 30 nm solenoid fiber into 'loops' or 'domains' of chromatin (Reeves, 1984). These domains are thought to consist of supercoiled stretches of DNA, representing 35 - 100 bp or more of DNA, that are attached to a supporting nuclear structure by specific non-histone proteins. This supporting nuclear structure has been referred to using a number of terms including matrix, cage, lamina, envelope, nuclear membrane and in metaphase chromosomes, scaffold (cited in Reeves, 1984). The additional folding of the chromatin fibers in metaphase chromosomes would condense the 30 nm fiber to such a degree that a compaction factor of 400 - 800 or more would be achieved (Lewis and Laemmli, 1982).

Interphase chromosomes are less compacted than metaphase chromosomes but are thought to possess a similar fundamental chromatin structure. Wigler and Axel (1976) have shown that metaphase and interphase nuclei chromosomes, digested with staphylococcal nuclease, retain the same basic subunit of the chromatin fiber. Supercoiled domains of chromatin appear to be an essential part of higher order chromatin structure in eukaryotic cells. Goodwin et al. (1985) have suggested from their studies that the

major structural feature of active genes responsible for DNase I sensitivity is dependent upon higher order chromatin structure involving the 30 nm chromatin fiber.

DNase I Sensitivity of Actively Transcribing Chromatin

To date, numerous studies have provided strong evidence correlating DNase I sensitivity with potentially transcriptionally active genes. The first results that clearly demonstrated that chromosomal subunits were preferentially digested by DNase I were reported by Weintraub and Groudine (1976). In this study they investigated the DNase I sensitivity of DNA coding for active and inactive β -globin gene sequences in a number of chicken tissues. The DNase I sensitivity of the β -globin gene sequences was first evaluated in adult chicken erythrocyte nuclei. The DNA of the erythrocytes was lightly digested with DNase I so that 10% of the DNA was acid-soluble. Preferential digestion of the globin DNA was monitored using a complementary DNA (cDNA) probe to globin messenger RNA (mRNA) that was isolated from adult chicken reticulocytes. The degree of DNase I sensitivity was then visualized by the percent hybridization of the cDNA probe to the remaining 90% of the purified DNA. Twenty-five percent of globin cDNA probe hybridized to DNase I treated DNA. Control conditions whereby reticulocyte nuclei were not digested with DNase I, resulted in 94% of the probe hybridizing to erythrocyte DNA. These results clearly showed that globin gene sequences which are actively expressed in chicken reticulocyte nuclei were preferentially digested by DNase I. Conversely, the same gene was found to be resistant to DNase I attack in oviduct

nuclei, where it is not transcriptionally active.

Weintraub and Groudine similarly assayed the DNase I sensitivity of the ovalbumin mRNA gene in chicken fibroblast cells. These cells do not produce ovalbumin. Ten to twenty percent of the DNA in the nuclei was digested and hybridization analysis revealed that the ovalbumin gene was not preferentially digested by DNase I. These results demonstrated that active genes were preferentially digested by DNase I and that this sensitivity is tissue-specific.

Actively transcribed genes were considered to be DNase I sensitive because of an altered chromatin configuration surrounding those genes. This was further demonstrated by staphylococcal or micrococcal nuclease studies (MN). Like DNase I, MN also distinguishes between exposed and non-exposed regions of DNA, preferentially cutting the linker DNA located between nucleosome cores (Reeves, 1984). Digestion of erythrocyte nuclei DNA with MN did not result in the preferential digestion of globin or ovalbumin sequences. The resistance of the active globin genes and inactive ovalbumin genes to this enzyme suggested that these genes were packaged into nucleosome-like particles. Since DNase I recognizes the internal features of nucleosomes, the sensitivity of globin genes to this nuclease suggested that they were conformationally different from inactive nucleosomes. Increased sensitivity of erythrocyte DNA to MN after trypsin digestion suggested that an altered histone conformation was also associated with the chromatin surrounding globin genes.

Similar studies by Garel and Axel (1976) essentially complemented and confirmed the observations made by Weintraub and Groudine. The DNase I

sensitivity of albumin genes in chicken oviduct nuclei and in liver nuclei was assessed using similar methods. More than 70% of the ovalbumin gene sequences were preferentially digested in chicken oviduct nuclei DNA that had been digested to a 10% acid soluble level. Ovalbumin gene sequences in liver nuclei, where they are not expressed, were not preferentially digested by DNase I. Again, treatment of oviduct nuclei with MN did not selectively digest the ovalbumin genes. Additional evidence implicating an altered chromatin configuration around actively transcribing genes was provided by cleaving individual nucleosomal subunits. The DNase I specific sensitivity was lost when the genes were cut into individual subunits; providing further evidence that DNA sequences sensitive to DNase I attack may be the result of a structural conformation of chromatin that is directly related to transcriptional activity.

This DNase I sensitive conformation is not just limited to endogenous genes but also includes exogenous proviral DNA sequences that have integrated in actively transcribing regions of chromatin. Panet and Cedar (1977) assessed the DNase I sensitivity of the Moloney murine Leukemia proviral (MuLV) DNA that had been integrated into the genome of Swiss mouse cells. Chromatin isolated from these cells was separated on the basis of MN sensitivity, resulting in a fraction that was less protected by DNA-binding proteins than the other. Each chromatin fraction was subsequently treated with DNase I and the viral specific sequences were located by hybridizing the chromatin fraction with a MuLV ³[H]-cDNA probe. The hybridization results indicated that more than 50% of the proviral sequences were selectively digested by DNase I and that the majority of the

sequences were located in DNA that was not protected by proteins. DNase I sensitivity was also related to gene activity in that proviral DNA sequences in virus-producing cells were more sensitive to DNase I attack than those sequences in non-virus producing cells. These sequences were also more DNase I sensitive in actively dividing cells than those cells arrested in G₀ phase of the cell cycle which only produce small amounts of the virus.

Although DNase I sensitivity is strongly correlated to actively transcribing genes, it is not an indicator of actual transcriptional activity. Garel et al. (1977) have demonstrated that DNase I sensitivity only reflects the potential for a gene to be transcribed. Three classes of genes known to be transcribed at significantly different rates in chicken oviduct cells were analysed by DNase I digestion kinetics. Ovalbumin genes were representative of actively transcribed genes; scarce mRNA genes represented a set of genes that were transcribed several orders of magnitude lower than that of the ovalbumin genes, and globin genes represented genes that were not actively transcribed at all. Oviduct nuclei were treated with DNase I and the digestion kinetics were monitored by the percentage of radioactively labelled cDNA copies of mRNA hybridizing to the nuclease digested DNA products. The ovalbumin gene sequences and the subset of mRNA genes were both characterized by similar DNase I sensitivities despite their differences in transcriptional activity. The inactive globin gene sequences were resistant to DNase I digestion; the rate of reassociation of globin cDNA was identical to that observed for sonicated, undigested nuclear DNA. These studies suggest that an altered chromatin configuration exists and is responsible for maintaining

the transcriptional potential of a given gene but its DNase I sensitivity does not reflect its actual transcriptional potential.

Chromatin Structure and Developmental Regulation

Differential transcription of genes allows for precise control of gene expression in developmental processes and differentiation. Transcriptional control of these genes could involve changes in chromatin structure and composition that may regulate the accessibility of different regions of DNA. Two fundamental classes of mechanisms have been proposed to be involved in the conversion of chromatin from an inactive to a transcriptionally active state (Reeves, 1984). One mechanism, the process of gene activation or commitment, makes a select subset of cellular genes available for transcription. The second mechanism induces and regulates the actual expression of potentially active genes. DNase I sensitivity studies have been extensively used to monitor changes in potentially active genes or active regions of chromatin during the process of development and differentiation.

Stalder et al. (1980) investigated possible mechanisms regulating hemoglobin 'switching' in developing chick embryos. In most vertebrates, there is a 'switch' in the type of hemoglobin (Hb) molecule that is produced by the red blood cell population during development. Early in embryonic development, fetal Hb is produced by 'primitive' red blood cells that are characterized by a distinct cellular morphology and specific cell markers. Adult red blood cells, on the other hand, display a very different cell morphology and possess adult-specific cell markers. Adult

red blood cells mainly produce adult Hb. A few restricted cells in the adult population are still able to produce minor quantities of fetal Hb and are known as F cells.

DNase I sensitivity was used to investigate Hb switching in developing chick embryos and to assess the chromosomal structure of embryonic and adult β -globin genes during erythropoiesis. DNA of red cell nuclei was mildly digested with DNase I to produce 10 - 15 kb fragments, purified and restricted with a variety of restriction enzymes. The DNA was separated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with adult specific β -globin cDNA or a genomic clone coding for both embryonic and adult β -globin chains. The DNase I sensitivity of the β -globin genes was monitored by the disappearance of specific restriction bands after nuclease digestion. In embryo-derived red blood cells, both adult and embryonic β -globin sequences were found to be very DNase I sensitive. However, in adult erythroid cells, only the adult β -globin gene was still DNase I sensitive, the embryonic gene was not. In control experiments, globin genes in brain and lymphoid cell-derived nuclei were assessed for DNase I sensitivity and were found to be resistant. These results demonstrated that the developmental switch from embryonic to adult β -globin gene expression was associated with an apparent change in chromatin structure.

The presence of a DNase I hypersensitive site contained in a DNA segment close to the 5' end of the chicken adult β -globin gene was also found to be developmentally correlated (McGhee et al., 1981). The hypersensitive site was observed in erythrocytes derived from day 14

chicken embryos which express the adult gene, but not in nuclei isolated from primitive erythrocytes of day 5 embryos. Elgin (1981) has suggested that DNase I hypersensitive sites are involved in cell commitment and gene activation during differentiation in a regulatory capacity.

Additional observations supporting a regulatory function for DNase I hypersensitive sites were made by Fritton et al. (1984). Different modes of regulation of the chicken lysozyme gene were associated with discrete DNase I hypersensitive sites. In immature chickens, the lysozyme gene is under steroid hormone control. The synthesis of lysozyme mRNA can be reversibly induced by administering or withdrawing estrogen. In the adult, the lysozyme gene is constitutively expressed in mature macrophages. Three hypersensitive sites are known to reside in the DNA sequences upstream from the promotor of the lysozyme gene. One of the DNase I hypersensitive sites in oviduct chromatin disappeared and reappeared with steroid hormone withdrawal and secondary induction, reflecting the transition from a potentially active state to an active state of the lysozyme gene.

States of cell differentiation are also characterized by differences in DNase I sensitivity. Plasmid pBR322, simian virus 40 (SV40) genomes and the herpes simplex virus type 1 thymidine kinase (HSV-1tk) gene in teratocarcinoma-derived stem cells and differentiated cells were assessed along with total chromatin DNA for DNase I sensitivity (Huebner et al., 1981). The pBR322 plasmid, which was not expressed in either stem or differentiated cell line, was more DNase I sensitive in stem cells than in differentiated cells. The SV40 genome, although not expressed at the protein level in stem cells, was also preferentially DNase I sensitive in

the undifferentiated cells. The HSV-1tk gene which was constitutively expressed in both stem and differentiated teratocarcinoma cells was the least DNase I sensitive in either cell type. In general, the chromosomal DNA of stem cells was more DNase I-sensitive than that of differentiated stem cells. Huebner et al. contended that the higher level of DNase I sensitivity of stem cell chromatin was due to a different configuration that was not characteristic of differentiated cells nor was it related to its transcriptional state. The frequency with which DNase I sensitivity has been associated with the potential activity of transcribing genes, developmentally regulated genes and undifferentiated cell lineages make chromatin structure a prime candidate as a possible regulatory mechanism involved in transcription.

DNase I Sensitivity and Methylation of Chromatin

A number of studies have provided evidence that transcriptionally active regions of chromatin that are DNase I sensitive usually contain DNA that is undermethylated. Expression of globin genes, herpes thymidine kinase gene, mouse methallothionein gene, adenoviral and immunoglobulin genes and a number of other genes have been observed to be accompanied by a decrease in methylation of cytosine at specific sites in or near the gene (Felsenfeld et al., 1983). Many of these genes have been found to be DNase I sensitive as well (cited in Reeves, 1984).

Evidence correlating hypomethylation of DNA with enhanced sensitivity was clearly shown by Weintraub et al. (1981). Levels of DNA methylation and DNase I sensitivity of the α -globin gene cluster were examined in the

developing chicken embryos. In 14 day chick embryos, the embryonic type α -globin or U gene is not expressed, instead adult globin gene sequences are transcriptionally active. Expressed regions of the α domain were found to be undermethylated and the non-expressed regions were methylated. DNA isolated from cells that did not express globin was also found to be highly methylated compared to the DNA derived from red cells. These results clearly demonstrated that expressed regions of the α -globin domain were undermethylated and non-expressed regions were methylated.

DNAse I sensitivity assessment revealed that the coding regions of the α -globin genes were more DNAse I sensitive than the non-coding regions. Sequences of the U gene, which was also not expressed in 14 day red cells, were found to be DNAse I insensitive. Examination of the U gene at the developmental stage where it is actively being expressed revealed a DNAse I hypersensitive site at the 5' position of the gene.

A similar study was reported by Groudine and Weintraub (1981) with the β -globin gene. DNA coding for the active adult and embryonic chicken globin genes was also found to be undermethylated and DNAse I sensitive, depending upon which set of genes were being expressed. Both studies suggested that undermethylation of DNA was positively correlated with DNAse I sensitivity. Due to the association of DNA methylation and gene activity, methylation has been implicated as part of a secondary mechanism regulating gene expression (Doerfler et al., 1982).

DNase I Sensitivity of Mitotic Chromosomes

The previously described studies assessed the DNase I sensitivity of genes by digesting DNA in interphase nuclei. Gazit et al (1982) investigated the DNase I sensitivity of active genes at a different stage of the cell cycle, that of mitotic metaphase. This was a particularly interesting approach in that this is the stage where genes are not actively being transcribed. The DNase I sensitivity of potentially active genes during metaphase was assessed by examining the nuclease sensitivity of exogenous murine leukemia virus sequences in monolayer culture cells. Previous experiments showed that these genes were transcriptionally active and 3 - 4 times more sensitive to DNase I than total nuclear DNA. Nuclei of mitotic cells were isolated and subjected to DNase I treatment until approximately 10% of the total DNA had been digested. The remaining DNA was hybridized to a MuLV-specific probe and the percentage of hybridization was compared to total DNA from unsynchronized cultures. The viral genes were found to be 3 times more sensitive to DNase I digestion than the total DNA. Cross-hybridizing endogenous mouse viral sequences, known to be inactive, were found to be relatively DNase I insensitive in metaphase nuclei of NIH Swiss mouse cells. These experiments suggested that the chromatin conformation of active and inactive viral genes, based upon their DNase I sensitivity, are the same in metaphase and interphase chromosomes.

It then became necessary to determine whether this DNase I sensitivity was maintained in all or a wide range of active endogenous genes in metaphase cells and not just limited to the exogenous viral genes. DNA of metaphase nuclei was nick translated according to the procedure

originally reported by Levitt et al. (1979). It was shown in this earlier study that active genes can be uniformly labelled by nick translating nuclei. The DNase I preferentially nicks the DNA at active regions with the adjacent DNA being replaced with radioactive nucleotides by DNA polymerase. Gazit and co-workers used this procedure to nick translate metaphase chromosomal DNA. To demonstrate that the DNase I sensitive regions of the metaphase chromosomes were equivalent to the sensitive regions of interphase nuclei, the nick translated metaphase chromosomal DNA was allowed to hybridize to total undigested DNA and to DNA isolated from nuclei that were minimally digested with DNase I. It was assumed that the DNase I digested DNA would consist mainly of DNase I-resistant sequences since the DNase I-sensitive sequences would have been digested. The nick translated, labelled chromosomal DNA did not hybridize well to the DNase I digested DNA but did to the total DNA still possessing DNase I-sensitive sequences. This suggested that the sensitive regions of metaphase DNA were equivalent to the sensitive regions of interphase DNA.

Gazit et al. (1982) then applied this same nick translation treatment to fixed preparations of mitotic chromosomes. This involved preparing slides of fixed metaphase chromosomes that were harvested from human fibroblast mitotic cells. The slides were pretreated with DNase I and then DNA polymerase and tritiated dinucleoside triphosphates were added. Labelled nucleotide incorporation was visualized by autoradiography. Grains were found to be distributed on both interphase nuclei and mitotic chromosomes and the level of labelling was proportional to the extent of DNase I treatment. Metaphase chromosomes represented cells in mitosis and

interphase nuclei were indicative of cells at all other stages of the cell cycle. Since both interphase nuclei and metaphase chromosomes were found to be DNase I sensitive, this suggested that the DNase I sensitive conformation surrounding genes was constant during all stages of the cell cycle regardless of the activity of the genes.

These studies have shown that DNase I preferentially digests genes that have been or are transcriptionally active. Combined endonuclease studies have attributed this selective digestion to an altered more accessible configuration of the chromatin surrounding the gene. Inactive chromatin, on the other hand, is characterized by a more closed configuration that protects the DNA from nuclease attack. Since this preferential DNase I-sensitive conformation is maintained in fixed mitotic chromosomes, DNase I has proved to be a useful structural probe for gene expression.

In situ Nick Translation of Mitotic Chromosomes

Using the ground work originally laid by Gazit et al. (1982), Kerem and co-workers (1983) developed the in situ DNase I directed nick translation technique to study further the DNase I sensitivity of fixed mitotic chromosomes. Their primary goals were to determine whether active genes retained their preferential sensitivity to DNase I and whether they could be mapped in situ. For this reason, they asked whether differences in the DNase I sensitivity of DNA from active and inactive X chromosomes could be detected in cells derived from Gerbillus gerbillus primary lung fibroblast cultures.

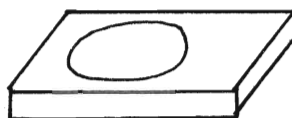
The reason for using the gerbil for the subject of the study becomes more evident when one cytologically examines the X chromosome of this species. The X chromosome is a composite chromosome, consisting of an 'original X' chromosome region that is flanked by autosomal segments that have been translocated to both sides. This compound X chromosome has been postulated to evolve from three translocations involving two pairs of autosomes and one pericentric inversion (Wahrman et al., 1983). Not only is this chromosome easily distinguished from the autosomes but it is also characterized by an allocyclic DNA replication pattern. The autosomal regions and a small band within the inactivated 'original X' chromosome replicate much earlier than the remaining regions comprising the 'original X' chromosome.

The procedure involved incubating slide preparations of fixed mitotic cells harvested from primary fibroblast cultures, with a nick translation mixture containing DNase I, ³[H]-TTP, cold dinucleoside triphosphates and DNA polymerase I. Labelled nucleotide incorporation was visualized by autoradiography. An outline of the procedure is shown in Figure 4.

Kerem et al. reported that all autosomes were labelled and that this label was symmetrically placed on sister chromatids. Individual chromosomes were characterized by labelled and unlabelled segments. The active X chromosome was reported as being labelled while the inactive X chromosome was less labelled in the original X chromosome region. The autosomal regions flanking the inactive X chromosome and the early replicating band within the original X chromosome were labelled. Seventy-nine percent of the cells examined demonstrated differential

Figure 4. Schematic diagram outlining the in situ nick translation procedure based upon that reported by Kerem et al. (1983) and (1984).

slide preparation of fixed mitotic cells



IN SITU NICK TRANSLATION MIXTURE

pancreatic DNase I

³

labelled nucleotide triphosphates ([H]-TTP or bio-16-dUTP)

cold nucleotide triphosphates

DNA polymerase I



visualization of nucleotide incorporation



autoradiography



giemsa staining



enzymatic detection

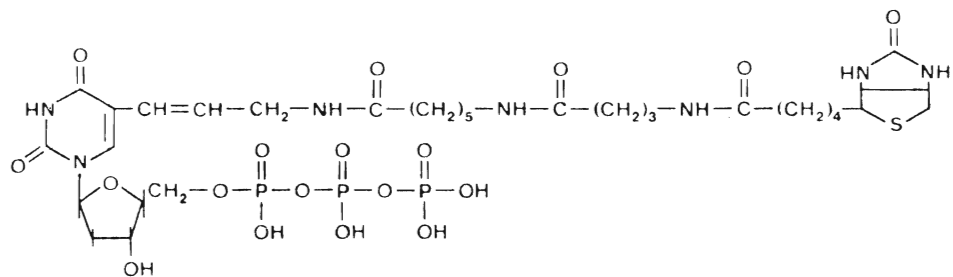
labelling of the two X chromosomes. It was suggested that the inactive X was characterized by a conformation which made it resistant to DNase I and therefore in situ nick translation could indeed distinguish active and inactive genes in metaphase chromosomes.

Use of Biotinylated-dUTP

Additional evidence indicating that transcriptionally active regions of mitotic chromosomes can be identified by in situ DNase I directed nick translation was presented in a subsequent study done by Kerem et al. (1984). This time the sensitivity and accuracy of the in situ nick translation procedure was increased using biotinylated dUTP and a specific detection and staining procedure in place of autoradiography.

The use of nucleotide analogues in place of radioisotopes as indicator probes is a fairly new and recent development. Langer et al. (1981) developed a non-radioactive labelled nucleotide, biotinylated deoxyuridine triphosphate (bio-dUTP), by covalently attaching a biotin moiety to the 5'-position of the pyrimidine ring of dUTP through an allylamine linker arm, as shown in Figure 5. Bio-dUTP effectively replaces thymidine triphosphate in a standard nick translation reaction catalysed by E. coli DNA polymerase I as it is an efficient polymerase substrate. Detection of biotinylated nucleotide incorporation is based upon the recognition of the biotin moiety by biotin binding proteins such as streptavidin, avidin or anti-biotin antibodies. Biotin, also known as vitamin H, was first isolated by Kogl (1935) from a liver concentrate known to contain growth factors for yeast. Its many features make it an ideal candidate for signal detection

Figure 5. Chemical configuration of biotinylated-16-deoxyuridine triphosphate (bio-16-dUTP). A biotin moiety has been covalently attached to the 5' position of the pyrimidine ring of dUTP through an allylamine linker arm.
(Reprinted from Enzo Biochemical Inc. 1985).

Bio-16-dUTP

5-[N-[N-(biotinyl)-ε-aminocaproyl]-γ-aminobutyryl]-3-aminoallyl]-deoxyuridine triphosphate

Molecular weight: 945

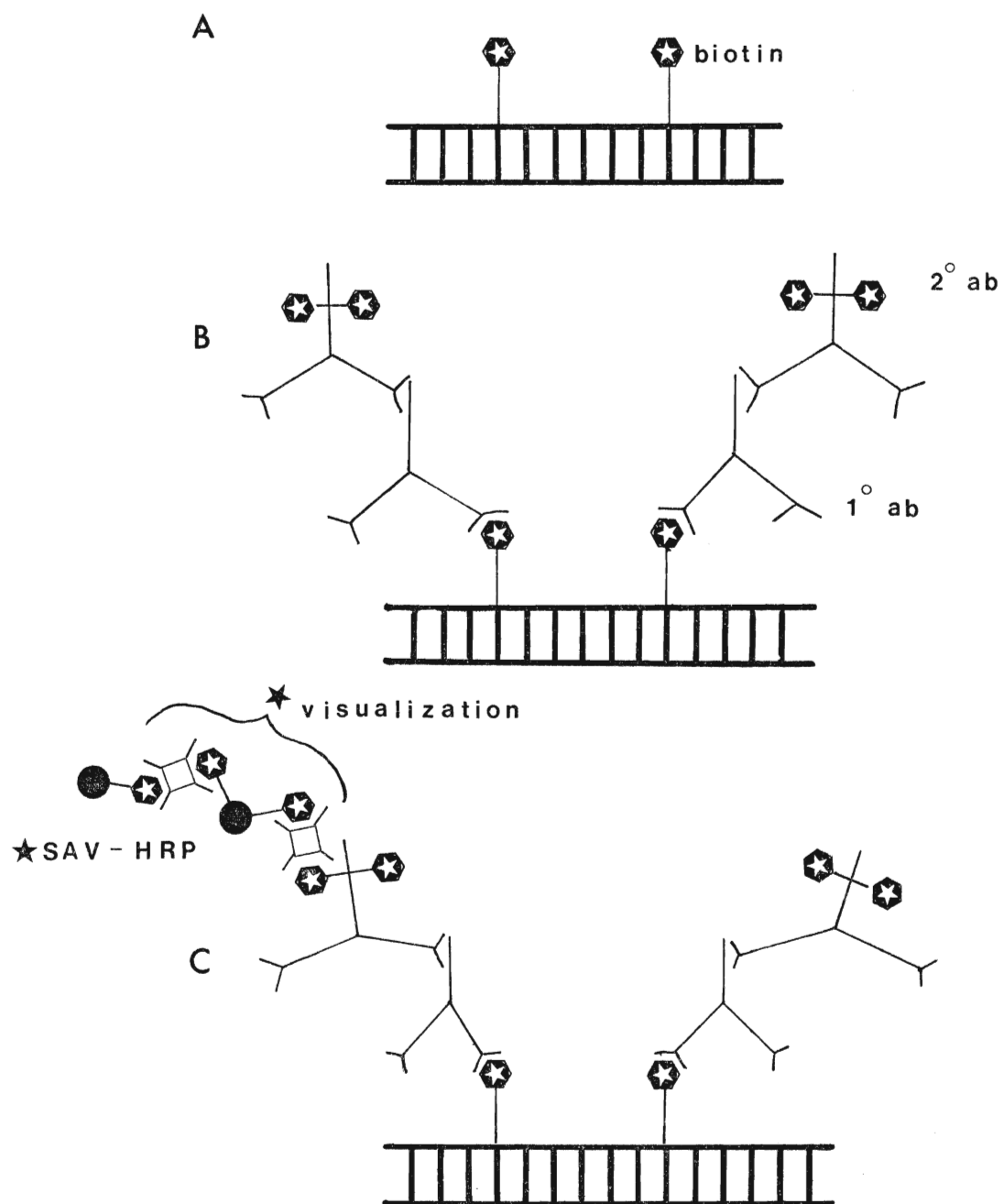
Formula: C₄₃ H₅₄ N₈ O₁₈ P₃ S (free acid)

and generation. Biotin is easily recognized by avidin, a 68,000 da glycoprotein known to exist in egg whites and is characterized by one of the highest binding constants known (cited in Langer et al., 1981). The avidin protein generally used is Streptavidin (SAV) a biotin-binding protein isolated from Streptomyces avidinii. Streptavidin is then coupled to an appropriate indicator molecule such as fluorescent dyes, electron dense proteins, enzymes or antibodies to form biotin-detecting complexes. Immunocytochemical detection involves using a biotin recognizing primary antibody and a biotinylated secondary antibody. The use of antibodies to detect biotinylated DNA insures against nonspecific binding to DNA and chromatin. This is then followed by enzymatic detection whereby streptavidin is complexed to the enzyme horseradish peroxidase (HRP). Streptavidin, characterized by multiple biotin binding sites, then specifically binds to the biotinylated secondary antibody. The use of the SAV-HRP enzyme complex has been shown to be a very sensitive indicator as streptavidin does not exhibit non-specific binding to chromatin (Hoffman et al., 1980). By adding an HRP enzyme substrate, diaminobenzidine tetrahydrochloride (DAB), the biotinylated DNA can be visualized by the formation of a brown precipitate on the chromosome. A figure depicting the immunocytochemical and enzymatic detection process is illustrated in Figure 6.

In situ Nick Translation of Mitotic Chromosomes Using Bio-dUTP

In situ nick translated human or chinese hamster ovary chromosomes demonstrated DNase I sensitive and insensitive regions, that resulted in a

Figure 6. Visualization of biotinylated nucleotide. (A) Incorporation of bio-16-dUTP by in situ nick translated DNA. (B) Primary antibody detection of biotinylated DNA by rabbit anti-biotin IgG, followed by secondary antibody detection by biotinylated goat anti-rabbit IgG. (C) Binding of secondary antibodies by Streptavidin-horseradish peroxidase enzyme complex (SAV-HRP) and visualization by diaminobenzidine tetrahydrochloride (DAB) staining.



specific dark and light banding pattern (D-bands) (Kerem et al., 1984). The dark D-bands represented DNase I-sensitive chromosomal segments and generally corresponded to light Giemsa bands (G-bands), a trypsin-Giemsa staining technique originally reported by Seabright (1974). It has been suggested by Holmquist (1982) that dark and light G-bands correspond to chromosomal clusters of replicons and that light G-bands replicate early in S-phase and dark G-bands replicate late in S-phase. The resistance of the G-bands to trypsin treatment also suggests that these regions of chromatin are more protected by protein than are the light bands. The light G-bands have also been implicated as actively transcribed regions of chromatin since cellular mRNA was shown to preferentially hybridize to these regions (Yunis, 1977). In addition to the production of light and dark D-bands, identifiable regions of inactive constitutive heterochromatin proved to be DNase I insensitive. The human Y chromosome is characterized by a large region of transcriptionally inactive constitutive heterochromatin located at the distal end of the long arm. This large block of heterochromatin was found to be DNase I insensitive while the euchromatic region, proximal to the centromere, was DNase I sensitive. The production of relatively consistent chromosomal banding patterns with the use of the biotinylated nucleotide, immunocytochemical and enzymatic detection in the in situ nick translation treatment has clearly enhanced the ability of the technique to detect potentially active genes on mitotic chromosomes. Evidence supporting the production of DNase I sensitive and insensitive bands was also reported by Murer-Orlando and Peterson, (1985), and Adolph and Hameister, (1985).

CHROMATIN STRUCTURE AND X-INACTIVATION

DNase I Sensitivity of Active and Inactive X Chromosomes

Conflicting results were reported by Murer-Orlando and Peterson (1985), and Adolph and Hameister (1985) regarding the DNase I-sensitivity of the active and inactive X chromosomes and of the heterochromatic region of the Y chromosome, respectively. Murer-Orlando and Peterson did observe that actively transcribed regions of human and mouse chromosomes were preferentially DNase I sensitive and that pericentric heterochromatic regions were DNase I-resistant but no differences were observed between the two X chromosomes. The inactive X chromosome was not distinguished from the other chromosomes as originally reported by Kerem et al. (1983). Adolph and Hameister also observed the production of DNase I-sensitive and insensitive bands but did not observe substantial differences in the DNase I sensitivity of euchromatin and heterochromatin of the Y chromosomes. Instead a boundary of preferential DNase I sensitivity was observed between the euchromatic and heterochromatic regions. However, centromeric regions of human chromosomes were generally not labelled. They were also unable to observe any differences between the active and inactive human and mouse X chromosomes. Adolph and Hameister postulated that the difference observed by Kerem et al. (1983) for the gerbil X chromosome could possibly be explained by the fact that small quantitative differences in DNase I sensitivity could be more clearly seen using autoradiography.

These conflicting views concerning the DNase I sensitivity of active and inactive chromatin in the form of euchromatin, constitutive or facultative heterochromatin only add ambiguity to the correlation between DNase I

sensitivity and genetic activity.

The conflicting results just described regarding the DNase I sensitivity of active and inactive X chromosomes were further enhanced by the findings of Riley et al. (1984). In this study, the DNase I sensitivity of specific DNA sequences encoding 3-phosphoglycerate kinase (PGK), associated with the X chromosome, was assessed. Nuclei were isolated from a variety of human cell lines and tissues and digested with increasing levels of DNase I. The purified DNA was treated with various restriction enzymes and analysed by Southern blot hybridization using a cloned cDNA PGK-specific probe. No differences were detected in the DNase I sensitivity of the PGK encoding sequences on either X chromosome except for a specific slightly sensitive site observed at relatively high levels of DNase I treatment. This slightly sensitive site was assumed to be on the active X chromosome since cell lines with increased numbers of inactive X chromosomes did not show an increase in the region of the chromatin that was sensitive. The restricted DNase I sensitive site was approximately 200 bp in size and located at the 3' end of the transcriptional unit. The establishment of DNase I hypersensitive sites at the 3' or 5' ends of genes has been suggested by Elgin (1982) to represent cell commitment and gene activation during differentiation. The findings of Groudine and Weintraub (1982) do not advocate the appearance of hypersensitive sites as a secondary consequence of either gene activation or transcription. At most, these sites are thought to be employed in a regulatory capacity by opening up short regions of chromatin so that important DNA sequences such as promoters, terminators, enhancer elements, origins of replication, the ends

of some chromosomes and regions required for specific gene rearrangements could become available for regulatory substances interactions (Reeves, 1984). It was concluded that except for this restricted region of enhanced sensitivity, chromatin configurations for PGK encoding sequences on the active and inactive X chromosomes were similar.

The results of Riley et al. (1984), Murer-Orlando and Peterson (1985), and Adolph and Hameister (1985) clearly contrast with the results originally reported by Kerem et al. (1983), that differences in the DNase I sensitivity of active and inactive X chromosomes could be detected, and reflect structural differences between the two chromosomes.

QUESTIONS ADDRESSED IN THIS STUDY

Possible mechanisms regulating X chromosome inactivation and transcriptional activities of tissues in the developing mouse embryo are of particular interest in this study. X-inactivation in the mouse embryo demonstrates regional and temporal patterns of inactivation that occur concomitantly with specific differentiative events but the exact timing and specific progenitor cells in which it occurs is still not clear. The pattern of X-inactivation with respect to parental origin is rather unique in that there is a random inactivation pattern observed in embryonic tissues but the paternally derived X^P chromosome is preferentially inactivated in extraembryonic tissues. Why this preferential inactivation of X^P occurs in the extraembryonic cell lineages and the possible regulatory mechanisms involved are still not clear. Recent studies have shown that the DNA from the inactive X chromosome is differentially modified from the DNA of the active X chromosome in embryo-derived and adult tissues. In extraembryonic tissues, however, the DNA from the inactive X chromosome was not differentially modified. A number of hypotheses have been proposed to try to explain the maintenance of X chromosome differentiation in mammals, one of which include DNA methylation. General levels of DNA methylation in embryonic and extraembryonic tissues have been assessed in an attempt to explain the possible causes of the differential modification observed of the DNA from active and inactive X chromosomes. The lowered levels of DNA methylation observed in extraembryonic tissues in which X-inactivation occurs suggest

that aspects of regulation dependent upon DNA modification may differ between the two lineages. The recent development of DNase I-directed techniques to detect transcriptionally active and inactive regions of mitotic chromosomes has attracted much attention. Kerem et al. (1983) reported that in situ nick translation could distinguish the active and inactive X chromosome of the gerbil on the basis of differing DNase I sensitivities. This would suggest that structural differences exist in the chromatin configuration of the active and inactive X chromosome, implicating chromatin structure as a possible mechanism regulating activity. Since extraembryonic tissues are generally undermethylated and characterized by preferential inactivation of X^P , the DNA of which is not differentially modified, the use of in situ nick translation would allow one to ask whether differences exist in the DNase I sensitivity of X^m and X^P in these tissues. This could shed some insight as to possible mechanisms involved in the regulation of X chromosome activity and whether tissues specific mechanisms exist. However, the recent controversy reported in the literature cast some doubt as to whether the inactive X chromosome in non-translocation type karyotypes could be distinguished by preferential DNase I sensitivity. The primary goal of this study was, therefore, to carry out a detailed evaluation of the potential of in situ nick translation for distinguishing differences in the DNase I sensitivity of active and inactive mouse and human chromosomes. At the same time, the ability of in situ nick translation to distinguish constitutive heterochromatin was also assessed, since this had also been the subject of some controversy. Finally, the ability of DNase I to detect overall

differences in chromatin structure in different embryonic tissues was assessed by in situ techniques. Gross differences in DNA methylation have been observed between different tissues in the mouse embryo and it is important to discover techniques for assessing whether these differences are accompanied by similar large differences in chromatin structure. This could result in a greater understanding of underlying mechanisms regulating overall levels of tissue specific gene activity and X-chromosome activity in the developoing mouse embryo.

In summary, the questions that were addressed in this study are as listed on the following page.

1. Can facultative heterochromatin of the inactive X chromosome be distinguished from the active X chromosome by in situ nick translation?
2. Can constitutive heterochromatin be distinguished in mitotic chromosomes by the same technique?
3. Can overall differences in DNase I sensitivity be detected between cells of different tissues by in situ techniques?

MATERIALS AND METHODS

A. CELL SPREADS AND TISSUE SOURCES:

CELL SPREADS DERIVED FROM CD1 MOUSE FEMUR BONE MARROW:

The femurs were sterilely dissected from a CD1 female mouse and placed into a plastic petri dish containing two ml of sterile complete MEM Alpha medium (α MEM) that was supplemented with 0.22% sodium bicarbonate, 5 % fetal calf serum, 5 % newborn calf serum, 5 mg/ml penicillin and 5.97 mg/ml streptomycin. The bone marrow was flushed out of the femurs using a 26 gauge, 1.25 cm needle and a 1 ml syringe with an additional two ml of complete α MEM. The marrow cell suspension was transferred to a tightly capped 10 ml glass test tube and incubated with 2 μ g/ml Colcemid (Gibco) for 1-1.5 hours at 37 °C. The cell suspension was then harvested according to the usual methods later described.

FIBROBLAST CULTURES DERIVED FROM 15.5 DAY MOUSE EMBRYOS:

Embryonic fibroblasts were cultured from 15.5 day CD1 X CD1 embryos using a modification of the procedure reported by McBurney and Adamson (1976). The embryos were sexed according to the protocol suggested by Vickers (1967). The amnion was removed from each embryo and carefully spread out on a precleaned dry slide. The tissue was then stained with 2 % Aceto-Orcein for 30-60 seconds before covering with a 22 X 60 mm coverslip. Amnion cell nuclei were observed under oil immersion (100 X magnification) and scored for the presence of sex chromatin located near the nuclear

membrane. The sex of the embryo was determined by the percentage of cells positive for sex chromatin. Female embryos have 67-90 % of the amnion nuclei positive for sex chromatin while male embryos have only 2-30 % positive. The sex of the embryo was also verified after harvesting by examining cell spreads for the absence of the Y chromosome in individual chromosome complements. The female embryos were then transferred to sterile 60 X 15 mm plastic petri dishes, minced with a pair of scissors and dissociated with 0.01 % trypsin (Sigma) in Ca⁺² -, Mg⁺² - free phosphate buffered saline (PBS) for 20 minutes at 37 °C. Trypsinization was stopped by the addition of an equal volume of complete α MEM. The cell suspension was then washed twice with equal volumes of complete α MEM to remove as much trypsin as possible. Single cell suspensions of approximately 1-2 ml were aliquoted to sterile 60 X 15 mm tissue culture dishes (Falcon 3002) and supplemented with an additional 3 ml of complete α MEM. The dishes were then incubated at 37 °C and 5 % CO₂ for 5-10 days or until near confluent fibroblast culture growth was obtained. The first medium change with complete α MEM was usually given 5-7 days after initial set-up and repeated every 2-3 days after that until cultures were ready to be harvested.

ISOLATION OF CELL SPREADS FROM 15.5 DAY EMBRYO DERIVED FIBROBLAST CULTURES:

Cell spreads were obtained from primary cell cultures that were treated with 0.12 μ g/ml Colcemid for 1-1.5 hours at 37 °C, 5 % CO₂ prior to harvesting. Fibroblast cells were trypsinized with 0.01 % trypsin in Ca⁺² -, Mg⁺² -free PBS for 5-6 minutes or until the majority of the rounded up,

dividing cells had lifted from the culture dish surface. The cell suspension was then transferred to a 10 ml glass test tube to be processed.

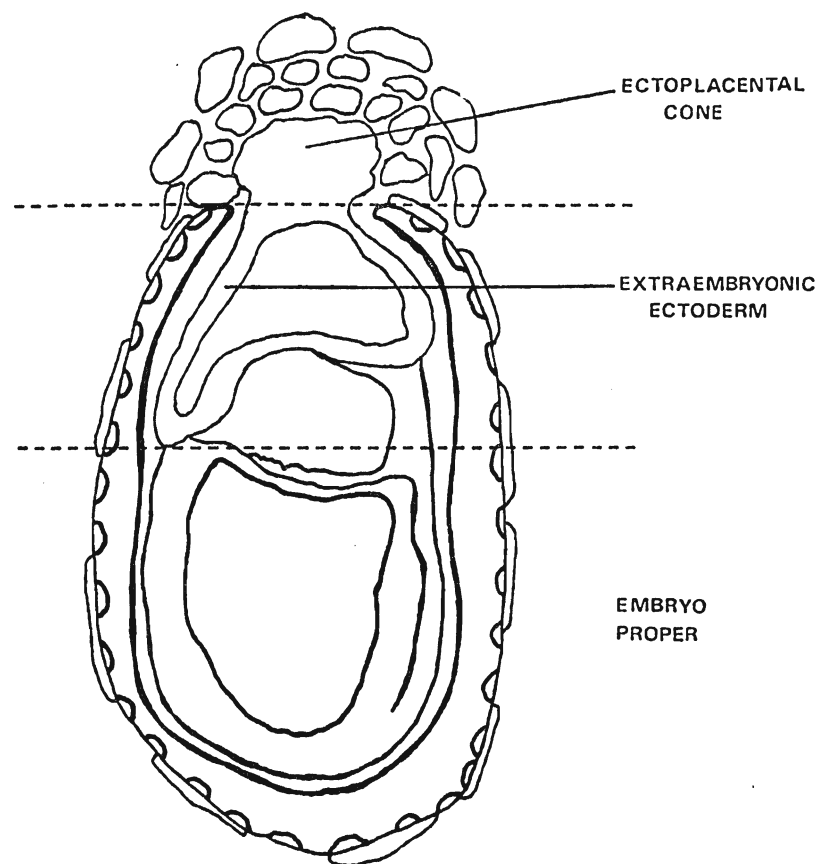
CELL SPREADS ISOLATED FROM HUMAN PERIPHERAL BLOOD:

A total of 5 ml of venous blood was collected from an adult female using a 21 gauge, 3.8 cm. needle and a 10 ml plastic syringe. The sample was then immediately transferred to a 10 ml test tube containing 0.5 ml of anticoagulant 3.8 % sodium citrate. From this, a volume of 0.5 - 1.0 ml. of whole blood was added to 4.5 ml of complete α MEM, 0.5 ml of 3.8 % sodium citrate and 2 % (v/v) Phytohemagglutinin (PHA, M form, Gibco) contained in a tissue culture flask (Falcon 3013 25 cm², 50 ml style). In addition to this, a plasma culture was also set up by allowing the plasma of the whole blood sample to separate out naturally at room temperature. A 2-3 ml volume of blood plasma was added to 9.0 ml of complete α MEM containing 1.0 ml of 3.8 % sodium citrate and 2 % (v/v) PHA. The cultures were all incubated in tightly capped tissue culture flasks for 72 hours at 37 °C. Prior to harvesting, the cultures were treated with 0.05 μ g/ml Colcemid for 30-40 minutes at 37 °C.

HARVESTING OF CELL SPREADS:

After Colcemid treatment, cell suspensions were pelleted by centrifugation at 800-1000 rpm for 6-10 minutes. The supernatant was then removed and the cell pellet was resuspended in 8-10 ml of 37 °C prewarmed hypotonic solution for 6-15 minutes. Bone marrow cell suspensions were treated with hypotonic 1 % sodium citrate while 15.5 day embryo derived

Figure 7. Diagram of a 7.5 day embryo in cross-section. The embryo is dissected into three distinct tissue types; ectoplacental cone (epc), extraembryonic ectoderm (ex), and embryo proper (emb). (Redrawn from Dean, 1985).



7.5 day Embryo

fibroblast cells and human lymphocytes were incubated with hypotonic 0.56 % KCl. After hypotonic treatment, the cell suspensions were again centrifuged using the previously described conditions. The supernatant was removed and the pellet resuspended using gentle vortexing. The cells were then fixed with cold, freshly prepared 3 : 1 methanol / glacial acetic acid by adding the first millilitre of fixative slowly in a dropwise manner, with sufficient mixing allowed in between. All cell suspensions were given a total of three fixative treatments of approximately 30 minutes each and/or stored overnight after the first fixative treatment. If the cell suspension was stored in fixative overnight, two subsequent fixative treatments of 30 minutes each were added the following day. The cell suspensions were stored at ^o-20 C throughout the whole fixative procedure.

ISOLATION OF CELL SPREADS FROM 7.5 DAY MOUSE EMBRYOS:

Embryos were obtained from CD1 females mated with males of the same strain and dissected at 7.5 days of gestation (day of plug was considered as day 0). The embryos were sterilely dissected into three distinct sections; embryo proper (emb), ectoplacental cone (epc) and extraembryonic ectoderm (ex) using No. 5 Watchmaker forceps (Kirby, 1971), (Figure 6). Extraembryonic ectoderm was isolated by incubating the dissected tissue in 2.5 % pancreatin in ⁺²Ca⁻, ⁺²Mg⁻ free Tyrodes solution containing 0.5 % ^otrypsin for 10 minutes at 4 C (Levak-Svajger et al., 1969). The endoderm layer was removed from ex by gentle trituration with a fine-blown Pasteur pipette. The three tissue sections were then incubated in petri dishes containing 2 ml of complete α MEM and 0.12 μ g/ml Colcemid for 1-1.5 hours

at 37 C, and 5 % CO₂.

HARVESTING OF CELL SPREADS FROM 7.5 DAY MOUSE EMBRYOS:

After Colcemid treatment, the dissected embryonic tissues were treated with prewarmed 37 C hypotonic 1 % sodium citrate in a plastic petri dish. The emb tissue was incubated for 5-15 minutes, epc for 2-15 minutes and ex for 2-4 minutes at 37 C. The tissues were then transferred to petri dishes containing 3 : 1 methanol/glacial acetic acid fixative and were stored overnight at -20 C before slides were made.

PREPARATION OF 7.5 DAY EMBRYOS FOR TISSUE SECTIONS:

Uteri containing 7.5 day embryos were sterilely dissected from CD1 females mated with CD1 males. The uterus was cut into individual sections containing the decidua encapsulated embryo. This was determined by a visible swelling of the uterus. These cut pieces were then fixed overnight in 3 : 1 methanol/acetic acid, stored at 4 C. The following day the embryos were drained of fixative and subjected to two, one-half hour washes in absolute alcohol. They were then treated with 1 : 1 absolute alcohol/ester wax (Gurr) for one hour at 60 C and finally incubated for two hours in pure ester wax alone. Embryos were embedded in tissue embedding molds that were rinsed with Mold Release. After the ester wax had hardened, the molds were stored at 4 C until used for sectioning.

Embryos were cut into 6 μ m sections using a Spencer 820 Microtome. The sectioned tissue was then placed onto a glass slide, precleaned with 70%

ethanol, by floating the section on a drop of distilled water and left at room temperature for 5 minute to ensure sufficient spreading. The slides were placed on a 37 C slidewarmer and allowed to dry completely. Once dried, they were immediately prepared for in situ nick translation or stored at 4 C until ready for use. Prior to in situ nick translation treatment, the mounted sections were rinsed in xylene followed by absolute alcohol for 5 minutes each to remove the wax. The slides were air dried before being used.

ISOLATION AND PREPARATION OF MITOTIC CELLS FOR in vitro NICK TRANSLATION:

Mitotic cells were obtained from near confluent, primary 15.5 day embryo derived fibroblast cultures treated with 0.04 ug/ml Colcemid (Gibco) for 2 hours at 37 C. The cultures were treated with cold, 0.1 % trypsin (Difco, 250:1) for 45 seconds to lift off the rounded-up, less firmly attached mitotic cells. Trypsinization was stopped by the addition of several volumes of complete MEM and the single cell suspension was transferred to 10 ml glass tubes kept on ice. The cells were washed twice with complete α MEM to remove as much of the trypsin as possible. The cells were collected by centrifugation; 800 rpm for 6 minutes at 4 C using an IEC refrigerated centrifuge. The supernatant was removed and the cell pellet resuspended. The cells were treated for 15 minutes at room temperature with a hypotonic solution consisting of a 1:5 dilution of culture media with distilled water. After centrifugation, the cells were resuspended in a 10 mM Tris-HCl (pH 7.5) buffer containing 10 mM NaCl, 3mM MgCl (Buffer A), 0.5 % Nonidet P40 (NP-40, Sigma) and 0.5 % Triton X-100.

The cells were left in the buffer for 30 minutes at 4 C. The suspension was again washed an additional two times in Buffer A without NP-40 and Triton X-100 before being pretreated with DNase I.

B. SLIDE MAKING PROCEDURES:

BONE MARROW, FIBROBLAST AND HUMAN LYMPHOCYTE CELLS

Cell spreads isolated from all sources were made by layering single drops of cell suspension on cold, precleaned, 70 % ethanol-covered or water-covered glass slides. Individual cells were further dispersed by gently blowing on the slide two or three times. The slides were dried using various methods depending on the source of the cell suspension. Bone marrow derived cell spreads were quickly flame dried over an open bunsen burner flame. Fibroblast cell spreads were either slowly flame dried or dried on a 40 C slidewarmer. Human lymphocyte cells were dried on a 40-60 C slidewarmer.

SLIDE PREPARATION OF CELL SPREADS FROM 7.5 DAY EMBRYOS:

Slide preparation involved placing individual pieces of tissue onto precleaned, dry slides and dissociating with 60 % acetic acid for 4-10 minutes. Individual cells of the tissue were further dispersed by blowing on the slides until all tissue remains were visibly spread out. The slides were either air dried or allowed to dry on a 40 C slidewarmer.

C. In situ NICK TRANSLATION

CELL SPREADS:

The standard protocol used for in situ DNase I - directed nick translation of cell spreads was initially based upon the procedure reported by Kerem et al, (1983). Slides were incubated in a nick translation mixture consisting of 50 mM Tris-HCl buffer (pH 7.9) containing 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 50 ug/ml bovine serum albumin, 10 U/ml DNA polymerase I (Boehringer Mannheim), varying concentrations of pancreatic DNase I, 4 μ M each of dATP, dCTP, dGTP and 0.3 μ M [³H]-TTP (40-50 Ci/mmol, NEN) or 3 μ M biotinylated-16-dUTP (Enzo Biochemicals). The reaction was carried out at various temperatures and incubation times. When [³H]-TTP was used as the labelled nucleotide the reaction was stopped by washing the slides with running tap water and rinsing them in ethanol. If biotinylated-16-dUTP was used, the reaction was stopped by rinsing the slides in PBS or 2 X SSC.

IN SITU NICK TRANSLATION VARIATIONS:

1. Temperature - reactions were carried out at room temperature or 14 °C
2. DNase I concentrations - 1, 4, 10, 15 and 20 ng/ml
3. In situ nick translation incubation times - 0 to 60 minutes

In situ NICK TRANSLATION OF 7.5 DAY MOUSE EMBRYO TISSUE SECTIONS:

Slides of 7.5 day embryo tissue sections were nick translated in situ using the nick translation mixture just described containing 4 ng/ml DNase I and 0.3 μ M [³H]-TTP. The reaction was consistently carried out at 14 °C for 40 minutes. Additional DNase I concentrations of 10, 15 and 20 ng/ml

were also tested using an incubation time of 40 minutes. Detection of nucleotide incorporation was as described in the following section.

DETECTION OF LABELLED NUCLEOTIDE INCORPORATION:

1. ³[H]-TTP

Incorporation of ³[H]-TTP was detected using autoradiography. In situ nick translated slides of cell spreads were dipped in liquid photographic emulsion (Kodak NTB2), diluted 2 : 1 water/emulsion. The slides were stored dry, in the dark, at 4 °C for up to a period of one month. The autoradiographs were developed with D-19 Kodak developer for 1.5 minutes and fixed with Kodak fixer for 5 minutes. The mitotic preparations were stained with 2 % Giemsa made up in a phosphate buffer, pH 6.8. Individual metaphases and interphase nuclei were examined for labelling with oil immersion (100 X magnification) using a Zeiss light microscope.

Dipped slides of 7.5 day embryo tissue sections were exposed for one week before being developed as previously described. The sections were then stained with the hematoxylin-eosin staining series (Appendix 1). Nuclei from embryo proper, extraembryonic ectoderm and ectoplacental cone were examined for labelling using a Zeiss light microscope.

2. Biotinylated-16-dUTP

a) Immunocytochemical Detection of Bio-16-dUTP Incorporation:

Immediately after in situ nick translation, the slides were rinsed in PBS at 4 °C and then rinsed again in PBS for five minutes at 32 °C. This was followed by a 5 minute incubation period in 0.1 % Triton X-100 in PBS at

room temperature. The slides were again rinsed in PBS for 5 minutes before being washed in 3 % Bovine Serum Albumin (BSA):PBS for 10 minutes. The slides were then incubated with the primary antibody, rabbit anti-biotin IgG (Enzo Biochemicals), diluted 1 : 100 in 2 % BSA - PBS. A 50 μ l aliquot was added to one half of each slide and incubated at 38 °C for 45-60 minutes before being washed three times in PBS for 5 minutes each wash. This was followed by a 10 minute rinse in 3 % BSA - PBS. The secondary antibody used was biotinylated goat anti-rabbit IgG (Enzo Biochemicals), also diluted 1 : 100 in 2 % BSA - PBS. Similar volumes were applied to each slide as previously described and incubated under the same conditions. The slides were subjected to three, 5 minute washes in PBS and a 10 minute rinse in 3 % BSA - PBS before being processed for enzymatic detection.

VARIATIONS IN IMMUNOCYTOCHEMICAL DETECTION AND CONTROL CONDITIONS:

Incubation of slides with:

1. primary antibody, SAV-HRP only
2. secondary antibody, SAV-HRP only
3. SAV-HRP only

b) Enzymatic Detection of Bio-16-dITP Incorporation:

Enzymatic detection of bio-16-dUTP incorporation involved adding a 100 μ l aliquot of Streptavidine-Bio-Horseradish Peroxidase (SAV-HRP) complex (Enzo Biochemicals), diluted 1 : 500 in PBS containing 1 % BSA, to each slide. These slides were then incubated at 38 °C for 60 minutes before being washed in 0.1 % Triton X-100 in PBS for 1 minute and then rinsed in PBS three times, each for 5 minutes. Peroxidase detection was carried out

according to the procedure suggested by the manufacturer. A 0.05 % diaminobenzidine (DAB) solution containing 100 μ l of 0.1 % hydrogen peroxide was freshly prepared in 10 ml of PBS. The DAB solution was liberally applied to each slide and allowed to stain for 5-40 minutes in the dark. The staining reaction was stopped by rinsing the slides well in distilled water to avoid salt crystallization and allowed to air dry. After air drying, the slides were examined with oil immersion (100 X magnification) using a Zeiss light microscope.

ANALYSIS OF IN SITU NICK TRANSLATED CELL SPREADS AND TISSUE SECTIONS

3 1. [H]-TTP

3
The net incorporation of [H]-TTP by interphase nuclei was quantitated by counting the number of silver grains per nucleus. A total of 20 nuclei were analysed per slide. Background labelling was taken into consideration by counting the number of silver grains found in an area of the slide approximately equivalent to an average-sized interphase nucleus. This value was subtracted from the grain count value obtained for each nucleus.

Metaphases chosen to be analysed were required to be of good quality that could be easily counted and karyotyped. Only metaphases with a complete chromosome complement were considered. In situ nick translated metaphases that were radioactively labelled with 20 silver grains or more were classified as being labelled. Chromosomes from each metaphase were also individually analysed for labelling and chromosomal banding patterns.

3
Analysis of [H]-TTP in situ nick translated 7.5 day embryo tissue sections was similarly quantitated by counting the number of silver grains

found on nuclei from the embryonic proper, extraembryonic ectoderm and ectoplacental cone. Again twenty nuclei were analysed for each tissue type per embryo with the mean value calculated for each tissue. Statistical analysis was done using the Apple Stats-Plus statistical package Wilcoxon Ranked Sum test ($P=0.05$).

2. Biotinylated-16-dUTP:

Cell spreads nick translated in situ with bio-16-dUTP were classified as being labelled by the presence of a brown stain found on chromosomes and interphase nuclei. Again metaphases of good quality and complete chromosome complements were chosen for analysis. Chromosomes from each metaphase were individually analysed for the presence of stain and chromosomal banding patterns.

KARYOTYPE PREPARATION :

1. Mouse Chromosomes

Mouse mitotic cells were karyotyped by cutting individual chromosomes out from a photomicrograph and arranged in homologous pairs, in decreasing order of size and according to similarities in labelling patterns (Nesbitt and Francke, 1973). The X chromosomes were identified as being the second largest acrocentric chromosomes of the complement.

2. Human Chromosomes

Human metaphase spreads analysed as being 46,XX were karyotyped according to the International System for Human Cytogenetic Nomenclature (1978). Photographs were taken of the cell spreads chosen for analysis

under oil immersion using a Zeiss light photomicroscope and Zeiss M35 camera set at automatic exposure. The cell spread was then karyotyped from a photographic print and cut out and arranged in homologous pairs according to the conventional human karyotype arrangement (ISCN, 1978). Since individual chromosomes could not be absolutely identified by their banding patterns produced by in situ nick translation, they were arranged into the seven groups, A-G, specified by the Denver classification, based upon descending order of chromosome size and position of the centromere. Any similarity in banding patterns between chromosomes was used as the final criterion for homologous pairing. The chromosomes were then individually compared to diagrammatic representations of human chromosomes characterized by G- and R-banding patterns (ISCN, 1978).

IN VITRO NICK TRANSLATION OF UNFIXED MITOTIC CELLS:

Unfixed mitotic cells were in vitro nick translated according to the procedures reported by Kuo and Plunkett, (1985). The hypotonic treated mitotic cells were resuspended in a 0.50 ml aliquot of Buffer A containing 1.0 $\mu\text{g/ml}$ pancreatic DNase I and pretreated for 10 minutes at 37 C. The cells were washed in a nick translation buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , 5 mM 2-mercaptoethanol (Buffer B), and then resuspended in a 0.25 ml aliquot of Buffer B containing 50 μCi ^3H -TTP, 3.0 μM each of dATP, dCTP and dGTP and 35 U DNA polymerase I (endonuclease-free, Boehringer Mannheim). The reaction was allowed to proceed for 1 hour at 14 C and stopped by the addition of an equal volume of 5 mM EDTA. The cells were washed twice in Buffer B alone and collected by centrifugation,

900 rpm for 12 minutes. The nick translated cell suspension was fixed by the addition of 3 methanol/1 glacial acetic acid. The fixed cells were centrifuged as previously described and some of the excess supernatant was removed. The cell button was resuspended in a small volume of remaining fixative and slides of mitotic cells were prepared according to the usual method described for fibroblast culture derived cells. After the slides were a dried on a 40 C slidewarmer, they were rinsed in 0.3 M NaCl/0.03 M sodium citrate, followed by a 70 % and 95 % ethanol rinse. The slides were allowed to air dry before being dipped in photographic emulsion as previously described. The slides were allowed to expose for 1 week before being developed by the usual methods. The cells were then stained with 10 % (v/v) phosphate buffered Giemsa stain (pH 6.8) for 3 minutes and were examined under oil immersion using a Zeiss light microscope for labelling.

RESULTS

3
IN SITU NICK TRANSLATION OF MITOTIC CELLS USING [H]-TTP

A. DETECTION OF LABELLING:

In situ nick translation of chromosomes derived from adult mouse bone marrow using the published treatment of 20 ng/ml DNase I and 5 minute incubation time at room temperature was not very successful. Slides were examined for labelling after 1, 2 and 3 weeks of exposure. Slides stored for 1 week were not labelled at all and slides stored for 2 weeks revealed 1-2 grains per metaphase. Slides stored for 3 weeks resulted in each chromosome being labelled with an average of 2-3 grains. Examination of interphase nuclei demonstrated a greater amount of labelling than the metaphase cell spreads but overall labelling was still variable and sparse in some areas of the slide. To establish a criterion for labelling, only those metaphases with a total grain count of 20 or more were considered to be labelled. Since the majority of the metaphases were labelled with less than 20 grains, these initial results were considered to be inconclusive. This led to the development of a modified in situ nick translation³ procedure using [H]-TTP.

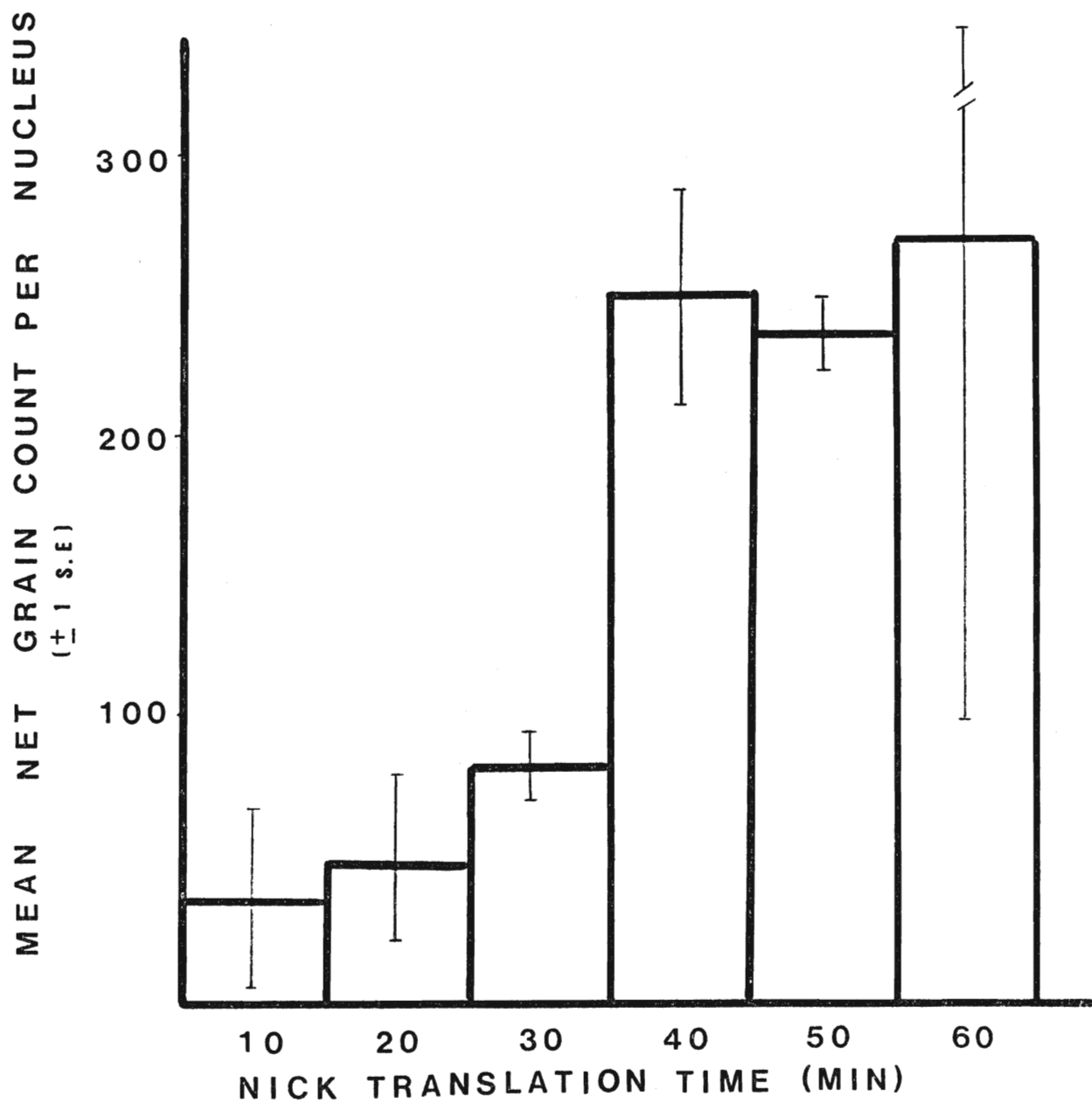
3
B. MODIFIED [H]-TTP IN SITU NICK TRANSLATION PROCEDURE:

To establish an effective in situ nick translation time and pancreatic DNase I concentration for the modified protocol, results were standardized by comparing mean net grain counts per interphase nuclei. Metaphases harvested from 15.5 day embryo-derived fibroblast cell cultures were nick

TABLE 1. THE EFFECT OF INCREASING DNase I TREATMENT TIME ON THE MEAN NET GRAIN COUNT OF MOUSE BONE MARROW DERIVED NUCLEI IN SITU NICK TRANSLATED WITH 4 NG/ML DNase I AND ^3H -TTP AT 14°C.

Nick Translation Time (min)	Slide	Number of Nuclei	Mean Net Grain Count per Nucleus ($\bar{X} \pm \text{S.D.}$)	Mean Net Grain Count per Nucleus
10	1	20	56.6 \pm 48.3	34.0
	2	20	11.8 \pm 12.9	
20	3	20	32.7 \pm 24.0	49.4
	4	20	65.9 \pm 12.9	
30	5	16	67.8 \pm 42.9	75.7
	6	20	83.6 \pm 68.5	
40	7	20	233.2 \pm 114.8	254.9
	8	20	276.5 \pm 108.8	
50	9	20	232.9 \pm 96.9	237.5
	10	20	229.9 \pm 89.6	
	11	20	249.8 \pm 142.2	
60	12	20	467.5 \pm 209.9	273.3
	13	20	142.3 \pm 57.0	
	14	16	210.0 \pm 116.5	

Figure 8. Histogram illustrating the effect of increasing DNase I treatment time on the mean net grain count of mouse bone marrow derived nuclei in situ nick translated with 4 ng/ml DNase I and ³[H]-TTP at 14 C.



translated in situ for 10, 20, 30, 40, 50 and 60 minutes at a controlled temperature of 14 C, using a DNase I concentration of 4 ng/ml. The results, shown in Table 1 and Figure 8, demonstrated a positive correlation between the mean net grain count per nucleus and increased treatment time. A mean grain count per nucleus of 254.9 ± 21.7 was obtained with an in situ nick translation time of 40 minutes. The extent of labelling was further quantitated by calculating the percentage of metaphases labelled for each incubation period. (The slides chosen for evaluation were identified by number and correspond to the same slide numbers previously listed in Table 1.) These values, presented in Table 2 and Figure 9, again indicated an increase in the percentage of metaphases labelled with increased in situ nick translation time. An incubation time of 50 minutes resulted in a maximum mean value of 66.2 ± 31.0 percent of the metaphases labelled. However, the extent of labelling still proved to be quite variable from slide to slide and within the slide despite the same experimental conditions, as can be seen from Table 2.

The degree of variability was clearly demonstrated by identically treating 10 slides of bone marrow derived cell spreads using a DNase I concentration of 4 ng/ml and an incubation time of 45-50 minutes at 14 C. In this experiment, the percentage of metaphases labelled ranged from 0-100 % on different slides, resulting in a mean value of 57.8 ± 32.3 % (Table 3).

To determine whether increasing DNase I concentration would result in an increased percentage of metaphases being labelled and a decrease in inter- and intra-experimental variability, concentrations of 10, 15, and

TABLE 2. THE EFFECT OF INCREASING DNase I TREATMENT TIME ON THE PERCENTAGE OF LABELLED METAPHASES AFTER IN SITU NICK TRANSLATION WITH 4 NG/ML DNase I AND ^3H -TTP AT 14°C.

Nick Translation Time (min)	Slide	Number of Metaphases Labelled per Slide	Mean % Metaphases Labelled
10	1	25/125 (20.0 %)	14.2
	2	11/130 (8.4 %)	
20	3	7/248 (2.8 %)	16.4
	4	55/184 (29.9 %)	
30	5	45/80 (56.3 %)	47.7
	6	50/128 (39.1 %)	
40	7	26/83 (31.3 %)	48.1
	8	68/105 (64.8 %)	
50	9	27/61 (44.2 %)	66.2
	10	140/159 (88.1 %)	

Figure 9. Histogram illustrating the effect of increasing DNase I treatment time on the percentage of mouse metaphases labelled after in situ nick translation with 4 ng/ml DNase I and ³H-TTP at 14 C.

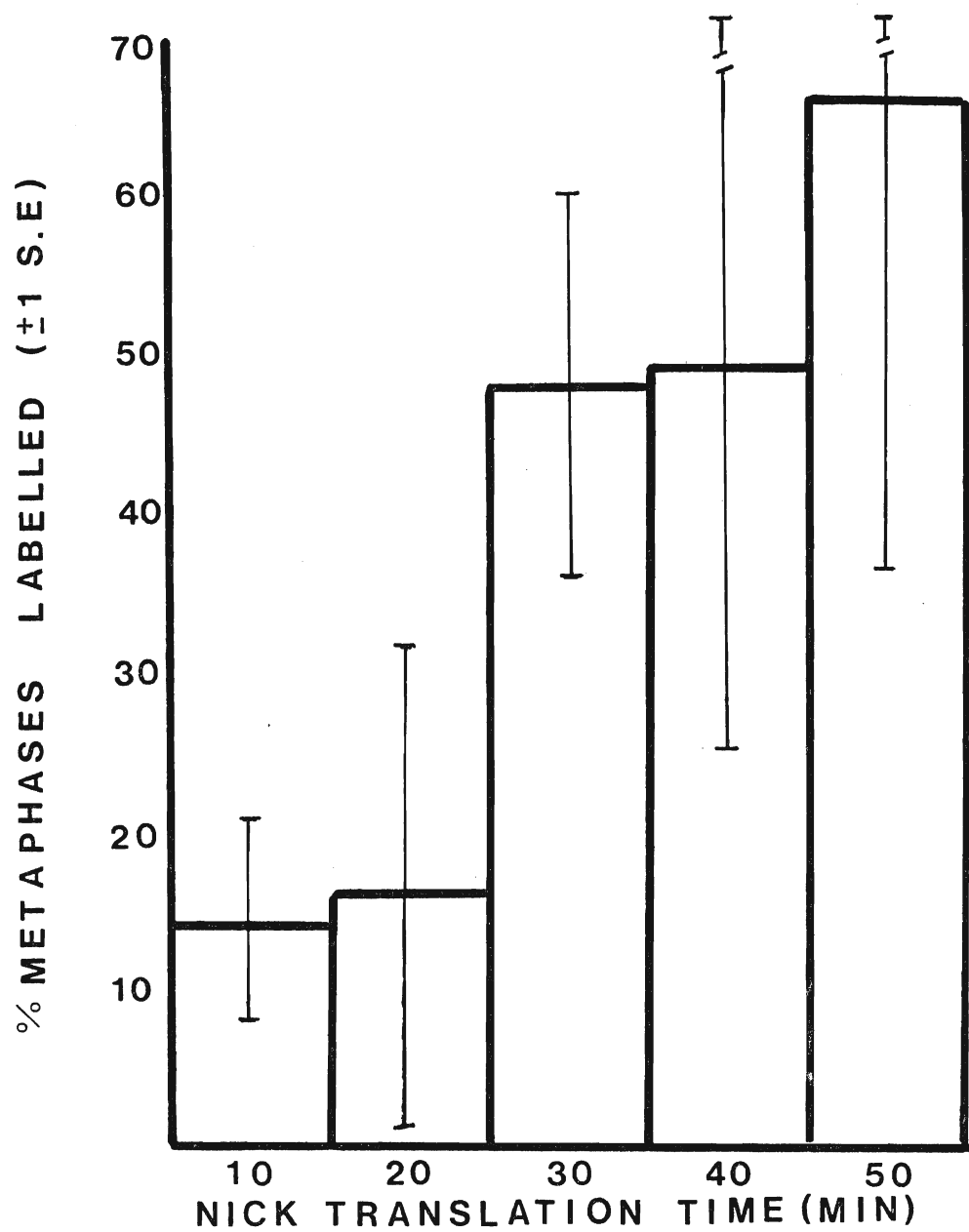


TABLE 3. THE PERCENTAGE OF FEMALE MOUSE BONE MARROW HARVESTED METAPHASES LABELLED WHEN IN SITU NICK TRANSLATED WITH 4 ng/ml DNase I, 0.3 μ M 3 H-TTP FOR 45-50 MINUTES AT 14° C.

Slide	Number of Metaphases Labelled per Slide	% Metaphases Labelled per Slide
1	0/27	0.0
2	22/37	59.5
3	11/20	55.0
4	15/15	100.0
5	20/21	95.2
6	4/11	36.4
7	16/16	100.0
8	7/18	38.9
9	9/18	50.0
10	6/14	42.9
$\bar{X} \pm \text{S.D.} = 57.8 \pm 32.3$		

20 ng/ml were tested using various time intervals. The cell spreads were harvested from female embryo-derived fibroblast cultures and the percentage of metaphases labelled by in situ nick translation are shown in Table 4. A DNase I concentration of 10 ng/ml with treatment times of 10, 20, 30 and 40 minutes resulted in an increase in the percentage of metaphases that was positively correlated with an increase in treatment time. A maximum value of 38.9 % metaphases labelled was obtained with a nick translation time of 30 minutes. Beyond this however, a decrease in the percentage of metaphases labelled (25.0%) was observed when the incubation time was increased to 40 minutes. Treatment with 15 ng/ml DNase I for 10, 20 and 30 minutes did not result in any metaphases being labelled while the 40 minute incubation time resulted in 66.7 % of the metaphases labelled (Table 4B). Metaphase chromosomes nick translated in situ for 5, 10, 15 and 20 minutes with 20 ng/ml DNase I did not result in any metaphases being labelled. This would explain why the initial in situ nick translation protocol using 20 ng/ml did not result in the labelling of chromosomes. The apparent decrease in labelling with increased DNase I concentration is not surprising since higher concentrations of DNase I and prolonged treatment time might be expected to result in nicking of DNA into progressively smaller pieces. These segments would then be lost from the slides with subsequent washings after the nick translation reaction, resulting in a decrease of observed label. Similar results were reported by Murer-Orlando and Peterson (1985) with human chromosomes that were in situ nick translated with increasing DNase I concentrations.

In summary, increasing DNase I concentrations did not result in a more

TABLE 4: THE EFFECT OF INCREASING DNase I CONCENTRATION AND TREATMENT TIME ON THE PERCENTAGE OF MOUSE FIBROBLAST CULTURE DERIVED METAPHASES LABELLED WHEN IN SITU NICK TRANSLATED WITH ^3H -TTP AT 14°C .

4A. 10 ng/ml DNase I

Nick Translation Time (min)	Slide	Number of Metaphases Labelled per Slide	% Metaphases Labelled per Slide
10	1	0/48	0.0
20	2	1/41	2.4
30	3	7/18	38.9
40	4	2/8	25.0

4B. 15 ng/ml DNase I

10	5	0/31	0.0
20	6	0/10	0.0
30	7	0/2	0.0
40	8	12/18	66.7

TABLE 4. THE EFFECT OF INCREASING DNase I CONCENTRATION AND TREATMENT TIME ON THE PERCENTAGE OF FEMALE MOUSE FIBROBLAST CULTURE DERIVED METAPHASES IN SITU NICK TRANSLATED WITH ^3H -TTP AT 14°C .

4C. 20 ng/ml DNase I

Nick Translation Time (min)	Slide	Number of Metaphases Labelled per Slide	% Metaphases Labelled per Slide
5	9	0/25	0.0
10	10	0/24	0.0
15	11	0/24	0.0
20	12	0/6	0.0

effective treatment to increase the percentages of metaphases labelled nor did it decrease the variability of the system. General trends from these experiments indicated that an increase in DNase I concentration resulted in a decrease in the percentage of metaphases labelled. It appeared that a DNase I concentration of 4 ng/ml and a treatment time of 40-50 minutes was the most effective for in situ nick translation of mouse mitotic cells and was used in all further experiments. A typical in situ nick translated cell is shown in Figure 10.

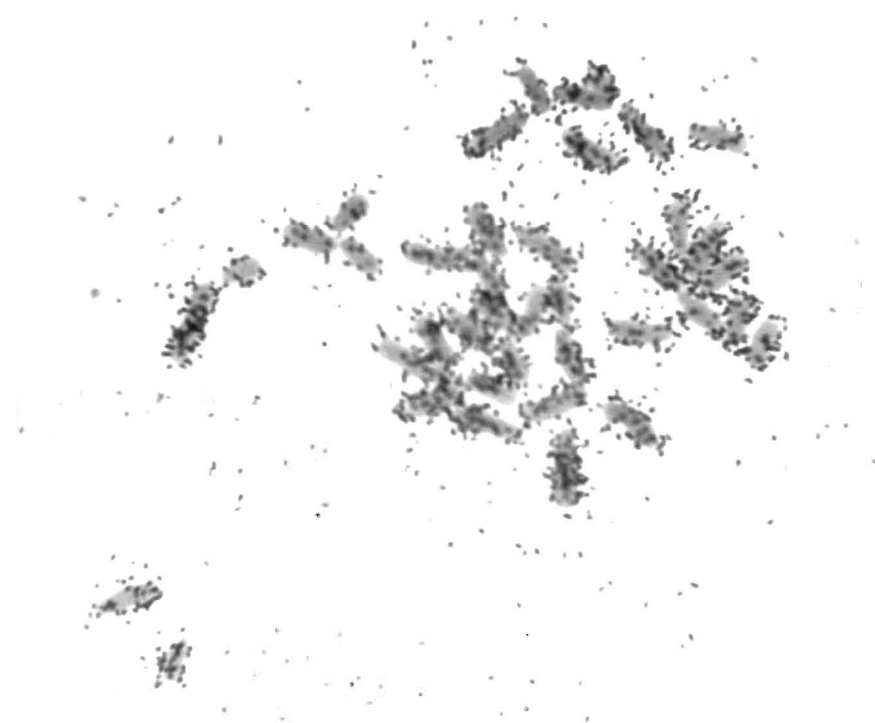
IN SITU NICK TRANSLATION OF CELL SPREADS USING BIOTINYLATED-16-dUTP:

A. Enzymatic Detection of Labelling

Mouse Chromosomes:

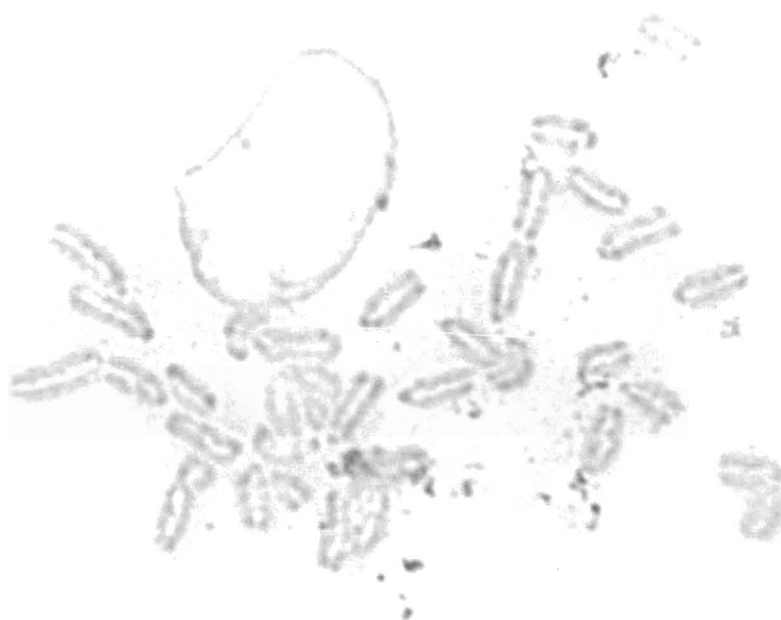
To try to further improve the specificity and to reduce variability of chromosome labelling by in situ nick translation so that chromosome banding patterns could be produced, a non-radioactively labelled biotinylated nucleotide was used in place of [H]-TTP³. Mitotic chromosomes harvested from female mouse fibroblast cultures were in situ nick translated using the same conditions described for the modified [H]-TTP³ procedure except that 3.0 μ M bio-16-dUTP was used as the labelled nucleotide. In this experiment, only direct enzymatic detection (SAV-HRP) was used to visualize nucleotide incorporation. A typical in situ nick translated metaphase is shown in Figure 11A. Figure 11B is a mitotic cell representative of those cells not treated with DNase I.

The percentage of metaphases labelled using this procedure were scored and presented in Table 5. The cell spreads that were treated with DNase I



0.01 μm

Figure 11. A. Mitotic cell harvested from 15.5 day female mouse embryo-derived fibroblast cells, in situ nick translated using 4 ng/ml DNase I and 3.0 μ M bio-16-dUTP and a 40 minute treatment time. Nucleotide incorporation was visualized by enzymatic detection only. B. Mitotic cell in situ nick translated without DNase I.

A**B**

0.01 μm

TABLE 5. THE PERCENTAGE OF FEMALE MOUSE FIBROBLAST CULTURE DERIVED METAPHASES LABELLED WHEN IN SITU NICK TRANSLATED WITH 4 ng/ml DNase I AND BIO-16-dUTP FOR 40 MINUTES AT 14°C AND VISUALIZED BY ENZYMATIC DETECTION.

Slide	% Metaphases Labelled	% Metaphases Banded
DNase treated	97.7 % (37/38)	10.5 % (4/38)
No DNase I	0.0	0.0
No Bio-16-dUTP	0.0	0.0

and bio-16-dUTP resulted in 97.7 % (37/38) of the metaphases being labelled. This is considerably higher than the mean 57.8 % value obtained using [³H]-TTP. Control conditions, whereby DNase I or the biotinylated nucleotide were omitted, did not result in any of the metaphases being labelled. In some cases, residual labelling of cytoplasm surrounding the cell spread was observed.

From these results, it can be stated that in situ nick translation of mouse chromosomes using bio-16-dUTP resulted in a greater percentage of metaphases labelled than the use of [³H]-TTP and was not limited by variable labelling.

B. Immunocytochemical and Enzymatic Detection of Labelling:

Human Chromosomes

To enhance chromosomal banding, immunocytochemical treatment of chromosomes with primary and secondary antibodies was introduced prior to enzymatic detection. Immunocytochemical detection involved using primary (rabbit anti-biotin IgG) and secondary (biotinylated goat anti-rabbit IgG) antibodies to enhance specificity.

To further define in situ nick translation conditions that would result in a more definitive chromosomal banding pattern, human female chromosomes were used. Human chromosomes have several advantages over mouse chromosomes in that they are well characterized with respect to morphology and chromosomal banding patterns and better quality cell spreads can be obtained. It was hoped that, should a banding pattern be produced by in situ nick translation, individual chromosomes like the X

chromosome could then be more easily identified. Chromosomes were harvested from a peripheral whole blood short term culture obtained from a female donor. Using a DNase I concentration of 4 ng/ml, mitotic chromosomes were nick translated for 10, 13, 15, 17, 20, 30 and 40 minutes at 14 C. Incorporation of label was visualized by immunocytochemical and enzymatic detection.

In situ nick translation conditions that resulted in an efficient level of labelling of human mitotic cells were again defined by scoring the percentage of metaphases labelled. These results are shown in Table 6. A nick translation time of 10 minutes did not result in labelling of chromosomes or interphase nuclei. All other treatment times, however, resulted in the labelling of all mitotic cells.

Control conditions where primary antibody, secondary antibody , DNase I or bio-16-dUTP were omitted were also tested (experiment 2, Table 6), using a standard incubation time of 30 minutes for all control conditions. When incorporation was visualized by enzymatic detection alone (omission of both primary and secondary antibodies), a reduction in labelling was observed in that the intensity of staining was much less. Omission of either the primary or secondary antibody, DNase I or the biotinylated nucleotide did not result in the labelling of any cell spreads except for the residual staining of surrounding cytoplasm.

General trends indicate that in situ nick translation using the biotinylated nucleotide was equally effective on human and mouse chromosomes. A more discrete labelling pattern was produced on human chromosomes with a much finer resolution using immocytochemical detection

Table 6. THE EFFECT OF INCREASING DNase I TREATMENT TIME ON THE PERCENTAGE OF HUMAN FEMALE METAPHASES LABELLED IN SITU NICK TRANSLATED USING 4 ng/ml DNase I AND 3.0 μ M BIO-16-dUTP AT 14°C AND VISUALIZED BY IMMUNOCYTOCHEMICAL AND ENZYMATIC DETECTION.

Nick Translation Time (min)	% Metaphases Labelled per Slide	% Metaphases Banded per Slide
EXPERIMENT 1		
20	100 (9/9)	44.4 (4/9)
30	100 (20/20)	40.0 (8/20)
40	100 (9/9)	44.4 (4/9)
EXPERIMENT 2		
10	0	0
13	100 (9/9)	44.4 (4/9)
15	100 (8/8)	11.1 (1/9)
17	100 (8/8)	11.1 (1/9)
no DNase I	0/4	0/4
no Bio-16-dUTP	0/4	0/4
no secondary antibody	0/5	0/5
no primary or secondary antibody	100 (2/2)	50.0 (1/2)

than could be obtained using autoradiography. Labelling produced by autoradiography is limited to the exposure of silver grains in the photographic emulsion by decaying ³[H]-TTP. Detection of labelling by way of precipitate formation is a much more sensitive and direct process.

ANALYSIS OF BANDING PATTERNS:

³ 1. [H]-TTP

Referring to Figure 10, examination of individual mouse chromosomes nick translated in situ with ³[H]-TTP seldom revealed any typical banding pattern and the extent of labelling appeared to be quite variable. Although clear banding patterns were not apparent, on some of the chromosomes grains appeared to lie in similar positions on both chromatids, producing a symmetrical labelling pattern. Certain chromosomes also appeared to be unlabelled at or near the centromere, regions that are known to be characterized by untranscribed constitutive heterochromatin. In other chromosomes, there was a greater concentration of silver grains located at the distal ends of the chromosome arms. This labelling distribution is consistent with the knowledge of the location of transcriptionally active genes of chromosomes. Many transcriptionally active genes are found to be located at the distal ends of chromosomes (Kuo and Saunders, 1977). Although a discrete chromosome labelling pattern was not produced using autoradiography, the results suggested that in situ nick translation treatment can distinguish regions of different transcriptional activity.

2. Bio-16-dUTP

A. Mouse Chromosomes:

A typical mouse mitotic cell that has been in situ nick translated with bio-16-dUTP and visualized with enzymatic detection only is presented in Figure 11A. The metaphase shown is characterized by minimal chromosomal banding with only the centromeric regions of many chromosomes escaping labelling. Referring back to Table 5, only 10.5 % (4/38) of the metaphases were characterized by variable chromosomal banding patterns.

B. Human Chromosomes

In situ nick translation conditions that resulted in an efficient level of labelling of human mitotic cells were also examined for chromosome banding patterns and the percentage of metaphases banded were scored and presented in Table 6. Treatment times of 13, 20, 30 and 40 minutes resulted in 40-44.4 % of the metaphases having chromosomal banding patterns. The fact that chromosomes treated for 15 or 17 minutes were not characterized by banding patterns is probably not related to the particular DNase I treatment but reflects the quality of the particular metaphases treated. Chromosomes that were shorter and more condensed, or impeded by cytoplasm were less likely to be banded than longer chromosomes. This is generally the case for any type of chromosome staining technique and therefore not necessarily a limitation of the assay. This could be the reason why the banding of mouse chromosomes was not as frequently observed as with human chromosomes since mouse chromosomes are much shorter.

The appearance of the chromosome bands as light and dark staining

bands is in agreement with the results reported by Kerem et al.(1984); Murer-Orlando and Peterson (1985), and Adolph and Hameister (1985). Kerem et al. termed these DNase I sensitive (dark) and insensitive (light) bands as D-bands.

Since constitutive heterochromatin was distinguished in mouse chromosomes by in situ nick translation, human chromosomes were also examined to see if the same phenomenon occurred. Chromosomes 1, 9 and 16 are known to possess regions of constitutive heterochromatin located at secondary constriction sites. These chromosomes were identified in four different cells, an example of which is shown in Fig 12. Examination of the secondary constriction sites revealed that constitutive heterochromatin escaped labelling. This was particularly noticeable with chromosomes 9 and 16 which are often characterized by relatively large blocks of constitutive heterochromatin.

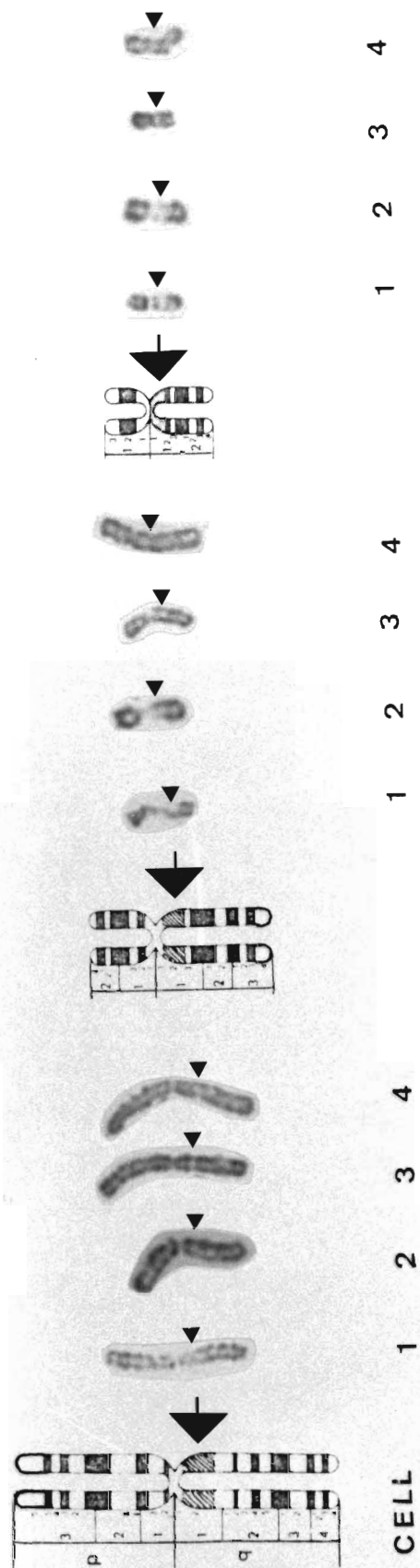
General trends indicate in situ nick translation using the biotinylated nucleotide was effective in distinguishing known transcriptionally active and inactive regions of chromosomes. Regions of constitutive heterochromatin in mouse and human chromosomes, considered to be transcriptionally inactive, were also distinguished by in situ nick translation. Regions of the distal ends of chromosomes, known to contain transcriptionally active genes were found to be more intensely labelled. In addition to this, a discrete dark and light banding pattern was produced on human chromosomes.

Figure 12. Analysis of human chromosomes 1, 9 and 16 from four mitotic cells in situ nick translated using 4 ng/ml DNase I and 3.0 μ M bio-16-dUTP for 30 minutes at 14 C. Nucleotide incorporation was visualized by immunocytochemical and enzymatic detection. Regions of constitutive heterochromatin are designated by arrows on diagrammatic representations of G-banded chromosomes

CHROMOSOME 16

CHROMOSOME 9

CHROMOSOME 1



ANALYSIS OF X-CHROMOSOME LABELLING:

3

1. [H]-TTP

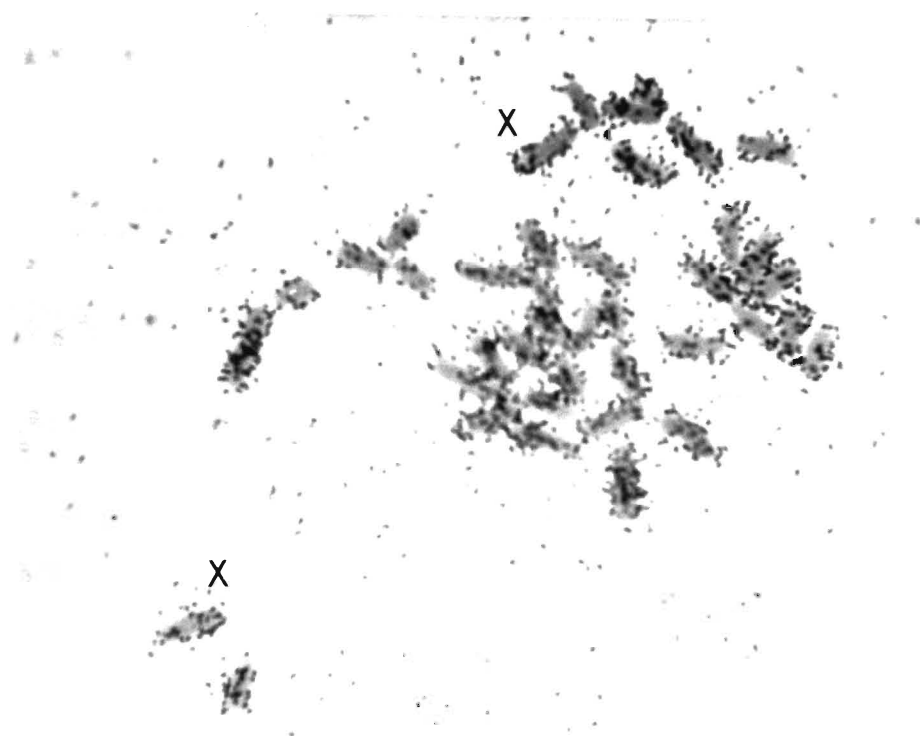
To determine whether the active and inactive X chromosomes were characterized by differences in DNase I sensitivity as detectable by in situ nick translation, the number of unlabelled chromosomes per metaphase was scored to see if a consistent number of individual chromosomes escaped labelling. Should the inactive X chromosome demonstrate DNase I insensitivity, it would escape labelling. This would consistently result in one unlabelled chromosome in each 40,XX complement. From the results presented in Table 7, it is clear that there was no predominance of metaphases exhibiting one unlabelled chromosome. Only 14 % of the metaphases examined had one unlabelled chromosome and these were often small chromosomes. It was not consistently observed that a large acrocentric chromosome which would be the morphology expected of an X chromosome escaped labelling.

To confirm that one of the X chromosomes did not escape labelling, a metaphase spread harvested from female, embryo-derived fibroblast cultures was karyotyped. Karyotype analysis involved cutting out the chromosomes from a photomicrograph and arranging them in pairs according to similarities in chromosome size and distribution of label. The X chromosome is the second largest acrocentric chromosome of the mouse chromosome complement (Nesbitt and Francke, 1973). The two chromosomes fitting that description are identified by the letter X in Figure 13. The distribution of silver grains over each chromosome appeared to be the same. Interestingly enough, one chromosome appears to stain more darkly with Giemsa than the other chromosome, a property that is characteristic of the

TABLE 7. ANALYSIS OF IN SITU NICK TRANSLATED FEMALE MOUSE BONE MARROW HARVESTED METAPHASES FOR THE NUMBER OF UNLABELLED CHROMOSOMES PER METAPHASE. (4 ng/ml DNase I, 0.3 μ M 3 H-TTP, 45-50 MINUTE TREATMENT TIME AT 14°C)

Slide	Number of Metaphases with Designated Number of Unlabelled Chromosomes per Metaphase					
	0	1	2	3	4	+ 5
2	11	3	4	8	4	15
3	9	5	1	1	0	1
4	0	0	1	0	3	0
5	0	0	0	0	0	1
8	2	2	0	0	0	1
Total (72)	22	10	6	9	7	18
Percentage (%)	30.5	13.9	8.3	12.5	9.7	25.1

Figure 13. Karyotype analysis of an in situ nick translated mouse mitotic cell, 40,XX, in situ nick translated using 4 ng/ml DNase I and $0.3 \mu\text{M}$ ^3H -TTP. X chromosomes are designated by the letter 'X'.



40, X X



inactive X chromosome (Tagaki and Oshimura, 1973). These findings collectively indicate that in situ nick translation of mouse mitotic cells using ³[H]-TTP did not result in the differential labelling of the active and inactive X chromosome. Consequently, no differences in their DNase I sensitivity can be inferred.

2. Bio-16-dUTP

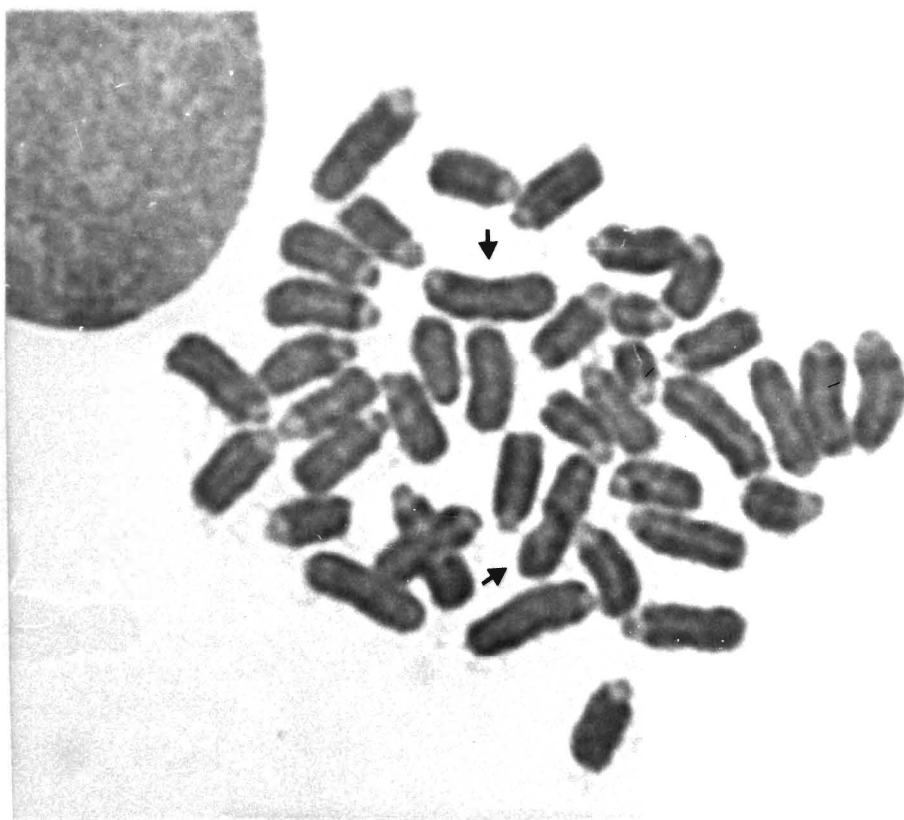
A. Mouse chromosomes

In situ nick translation of mouse chromosomes with bio-16-dUTP resulted in the labelling of all chromosomes of the complement. No chromosome consistently appeared to be any less labelled than any of the other chromosomes of the complement. Karyotype arrangement of the chromosomes according to size revealed that the second largest acrocentric chromosomes, representative of the X chromosomes, were equally labelled (Figure 14). From these results, it can be said that in situ nick translation using bio-16-dUTP did not distinguish the inactive X chromosome from the active X chromosome in the mouse.

B. Human Chromosomes

Human metaphases were also examined for possible differences in the labelling of the two X chromosomes. Figure 15 is a banded metaphase spread that has been karyotyped and arranged according to the Denver classification (ISCN, 1978). The chromosomes were arranged in seven distinct groups, A-G, on the basis of centromeric position and overall chromosome length. The final criterion for homologue pairing was

Figure 14. Karyotype analysis of an in situ nick translated mitotic cell, 40 XX, isolated from 15.5 day female mouse embryo-derived fibroblast cultures (4 ng/ml DNase I, 3.0 μ M bio-16-dUTP, 40 minute treatment time at 14^o C, enzymatic detection). The X chromosomes are designated by the letter 'X'.

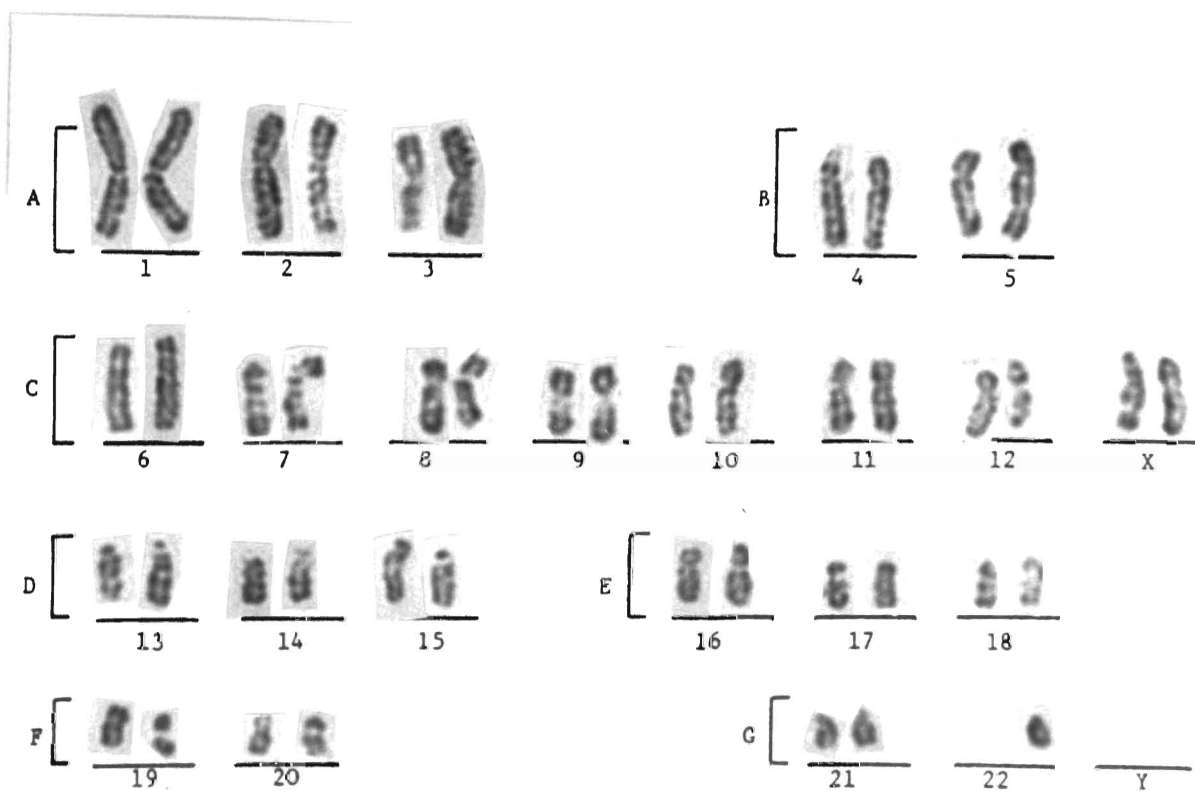
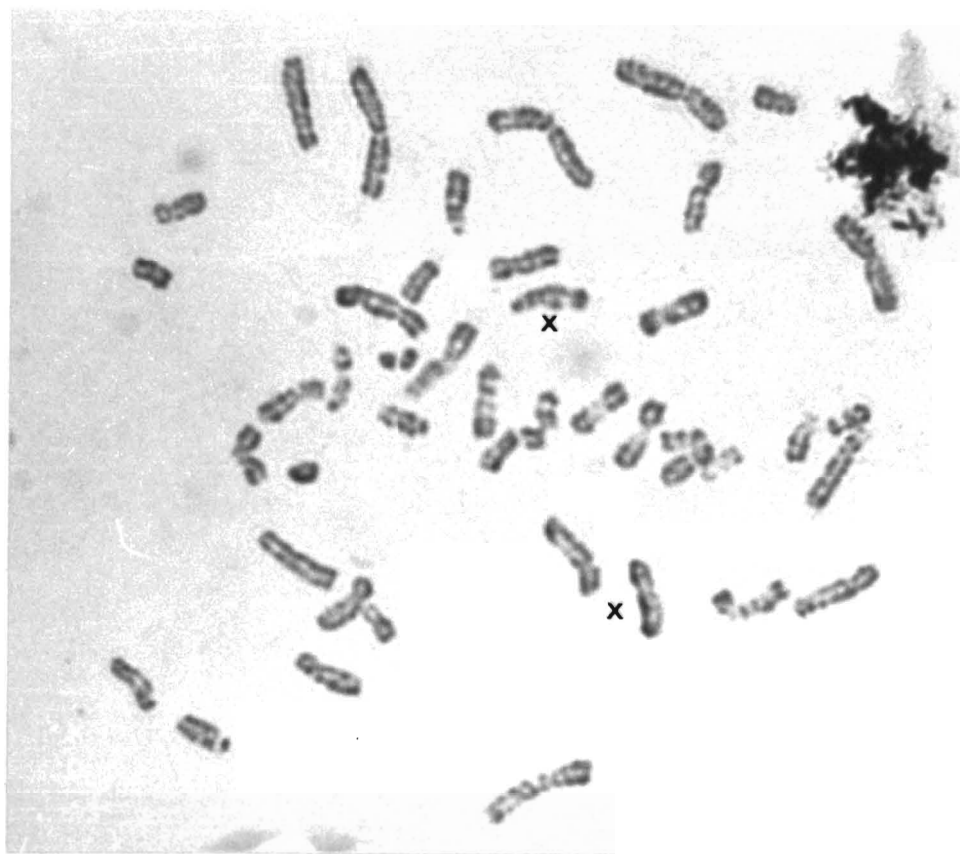


40, X X



X X

Figure 15. Karyotype analysis of a human mitotic cell, 45 XX, -G,
harvested from whole peripheral blood cultures and in situ nick
translated with 4 ng/ml DNase I and 3.0 μ M bio-16-dUTP for 30
minutes at 14 C. Nucleotide incorporation was visualized by
immunocytochemical and enzymatic detection.



similarities in chromosomal banding patterns. The X chromosomes, identified as the second largest chromosomes of the C group, were both equally labelled. This supported the observations seen in mouse mitotic cells where no differences were detected in the DNase I sensitivities of the X chromosomes using either ³[H]-TTP or bio-16-dUTP. Facultative heterochromatin in the form of the inactive X chromosome could not be distinguished by in situ nick translation in either mouse or human mitotic cells.

C. 7.5 Day Mouse Embryo Chromosomes

To determine whether the inactive X chromosome could be distinguished in embryonic and extraembryonic tissues of the 7.5 day female mouse embryo, mitotic cells were isolated from embryo proper, extraembryonic ectoderm and ectoplacental cone. The metaphases were in situ nick translated as described for human mitotic cells using an incubation time of 30 minutes. Bio-16-dUTP incorporation was visualized by immunocytochemical and enzymatic detection. The percentage of metaphases labelled and banded were scored and presented in Table 8. For all three tissue types, all cells in situ nick translated with 4 ng/ml DNase I were labelled and the percentage of metaphases banded ranged from 71.4 - 100.0 %. No single large acrocentric chromosome representative of the X chromosome consistently escaped labelling. Figure 16 A, B and C are metaphases harvested from 7.5 day embryo-derived tissues, extraembryonic ectoderm and ectoplacental cone tissue, respectively. The results observed in 7.5 day embryo tissues were similar to that observed with 15.5 day embryo-derived fibroblast culture

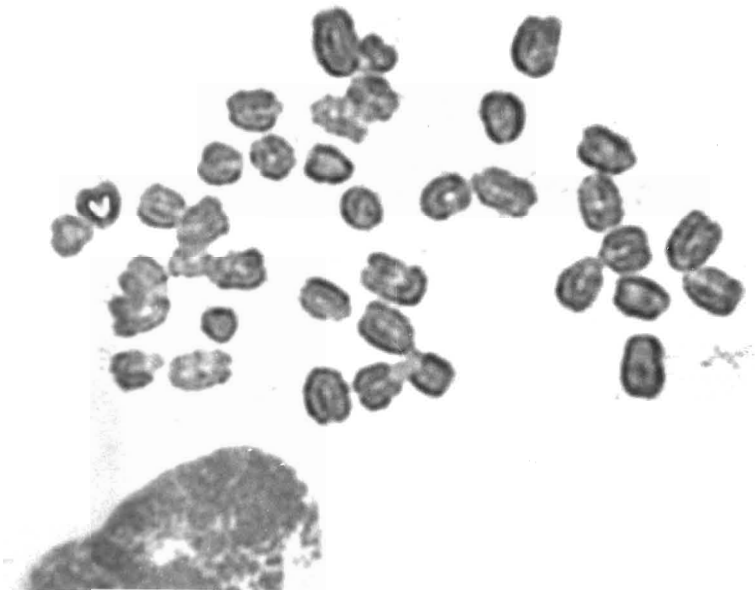
TABLE 8. THE PERCENTAGES OF METAPHASES, DERIVED FROM 7.5 DAY MOUSE EMBRYONIC TISSUE (EMB), EXTRAEMBRYONIC ECTODERM (EX) AND ECTOPLACENTAL CONE (EPC), LABELLED BY IN SITU NICK TRANSLATION USING 4 ng/ml DNase I AND 3.0 μ M BIO-16- dUTP.

Tissue Type	Slide	% Metaphases Labelled	% Metaphases Banded
emb	1	100 (6/6)	100 (6/6)
ex	2	100 (14/14)	71.4 (10/14)
	3	100 (2/2)	100 (2/2)
epc	4	100 (4/4)	75.0 (3/4)

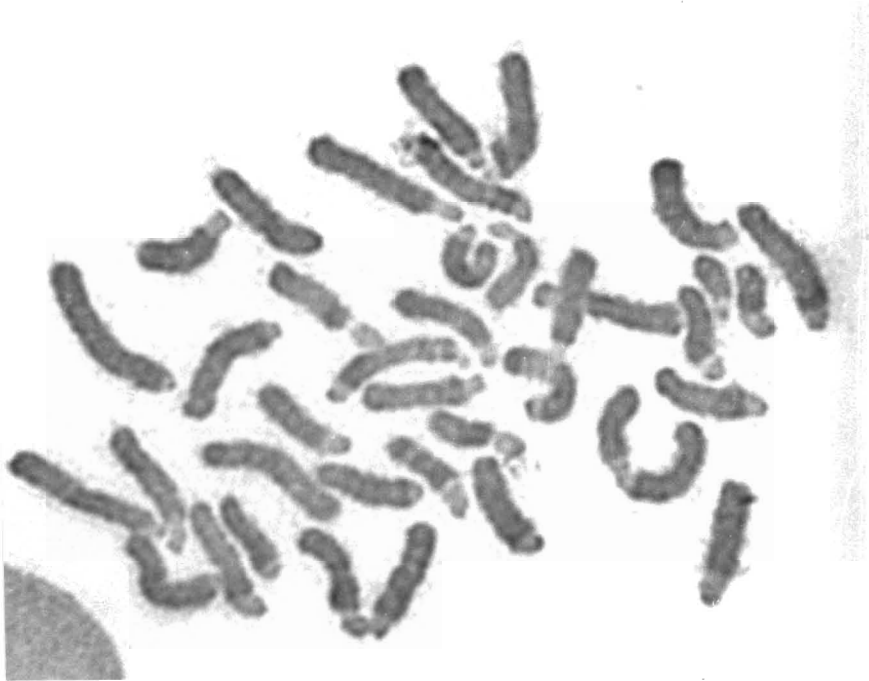
Figure 16. Mitotic cells harvested from 7.5 day embryo tissues, in situ nick translated using 4 ng/ml DNase I and 3.0 μ M bio-16-dUTP and a 30 minute treatment time. Nucleotide incorporation was visualized by immunocytochemical and enzymatic detection.

- A. Embryo proper
- B. Extraembryonic ectoderm
- C. Ectoplacental cone (see following page)

A



B





0.01 μ m

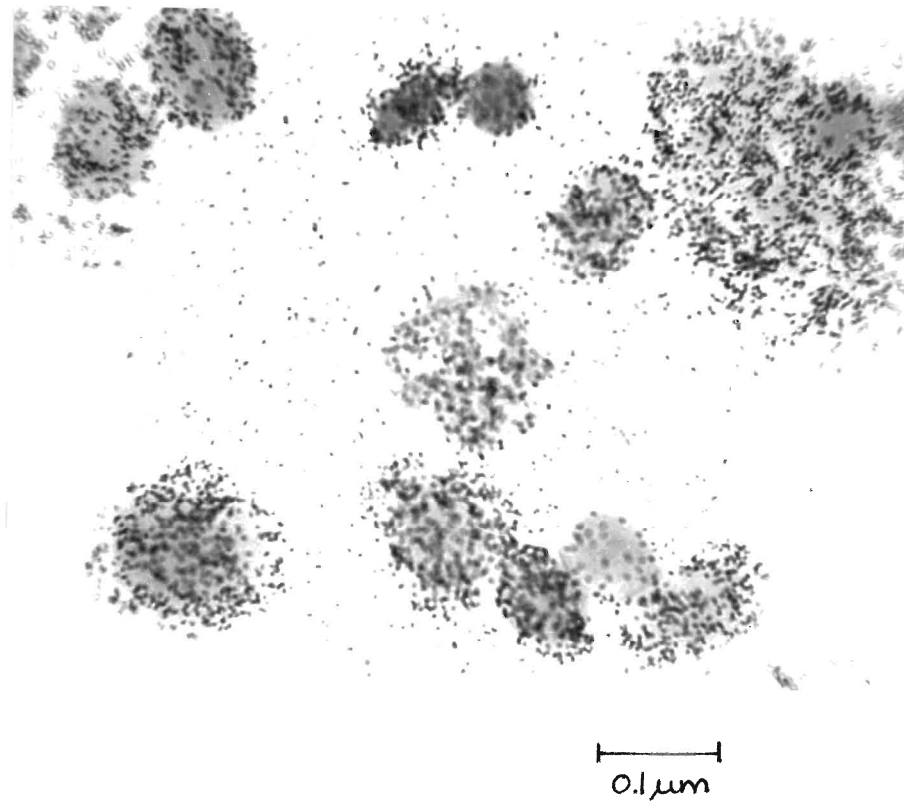
mitotic cells, adult mouse bone marrow derived cells and adult human lymphocyte derived mitotic cells. Constitutive heterochromatin localized at the centromeric regions was not labelled but no differences in the DNase I sensitivity of the active and inactive X chromosomes could be detected by in situ nick translation in tissues of either embryonic or extraembryonic origin.

IN VITRO NICK TRANSLATION OF MITOTIC CELLS

The technique of in situ nick translation has the disadvantage that chromosomes are fixed with methanol/glacial acetic acid, which may alter the native chromatin configuration (Riley et al., 1984). To assess whether fixation altered the DNase I sensitivity of chromatin to DNase I, unfixed mitotic cells were in vitro nick translated using a modification of the procedure reported by Kuo and Plunkett (1985). The distinct advantage of using this procedure is that the chromatin is still in an unfixed state and bound by intact nuclei while being subjected to DNase I treatment. This would allow one to assess the DNase I sensitivity of chromatin in a relatively natural configuration.

Cells isolated from 15.5 day mouse embryo-derived fibroblast cultures, were pretreated with 1 µg/ml DNase I for 10 minutes at 37 C and then nick translated for 1 hour at 14 C. After the nick translation reaction, the cells were fixed in 3 parts methanol / 1 part glacial acetic acid and slides of metaphase chromosomes were prepared. Nucleotide incorporation was visualized by autoradiography.

Figure 17 is a typical representation of interphase nuclei and mitotic



cells that have been nick translated in an unfixed state. It can be seen that the majority of nuclei are labelled, an observation that was consistent for all areas of the slide. No variability was seen between certain regions of the slide as was the case with the in situ nick³ translation treatment using [H]-TTP.

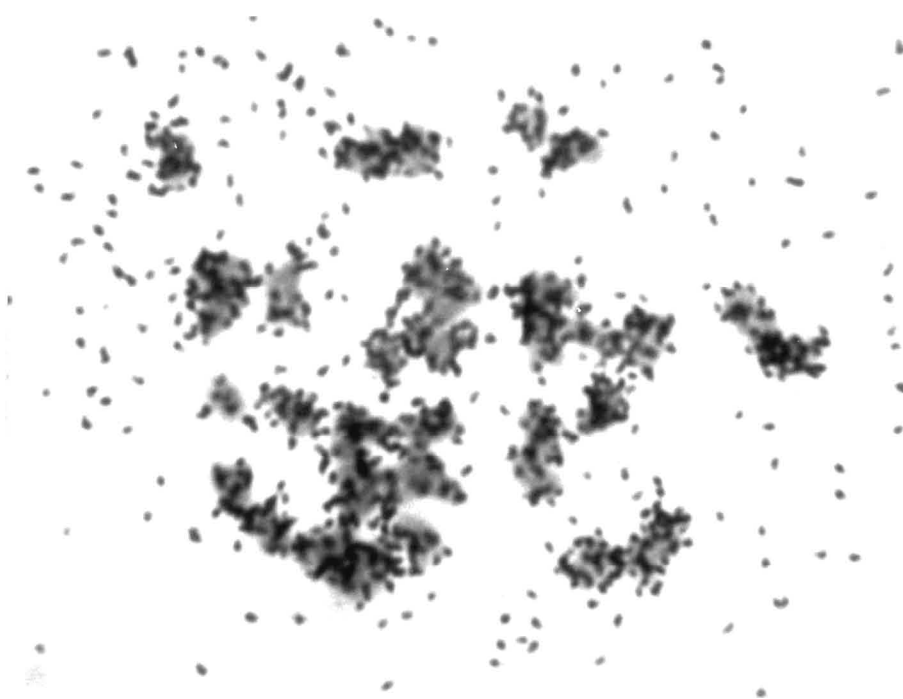
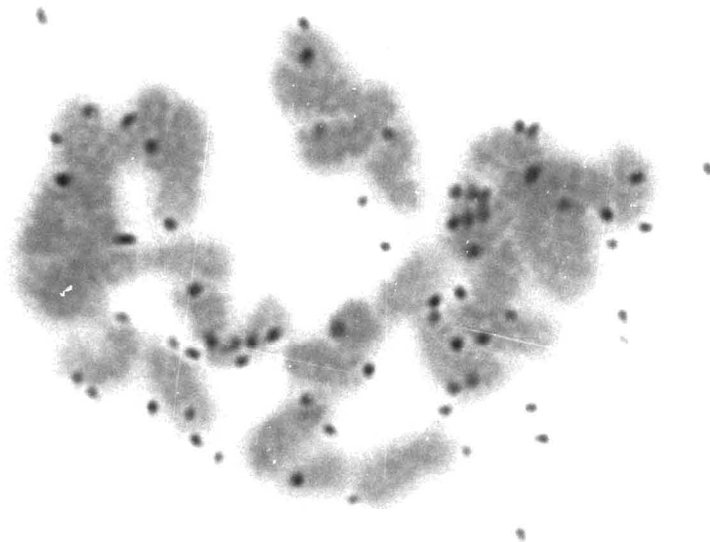
Figure 18A is a typical mitotic cell that has been nick translated with DNase I and Fig 18B is a cell spread that has been nick translated under control conditions without DNase I pretreatment. A distinct difference in the density and distribution of label can be seen between the two mitotic figures. The DNase I treated cell appears to be completely labelled. Because of the number of overlapping chromosomes, a definitive count could not be made. However, no one chromosome fitting the description of the mouse X chromosome appeared to escape labelling.

From this study, it can be concluded that in vitro nick translation of unfixed mitotic cells resulted in the labelling of interphase nuclei and mitotic cells, producing a pattern of labelling similar to but less variable than that achieved by in situ nick translation. One of the limitations of this procedure is that chromosome morphology and quality of cell spreads are not as well maintained as with in situ nick translation. The fixing of chromatin does not, however, appear to significantly alter the differentiated DNase I sensitivity of chromosomes.

OVERALL DNASE I SENSITIVITY ASSESSMENT OF SPECIFIC EMBRYONIC TISSUES:

The development of a technique that would allow one to assess overall levels of DNase I sensitivity in embryonic tissues was approached using in

- Figure 18. A. Labelling distribution of a mitotic cell in vitro nick translated in an unfixed state using a 10 minute, 1 $\mu\text{g/ml}$ DNase I pretreatment at 37 °C followed by a 1 hour nick translation reaction at 14 °C.
- B. Mitotic cell nick translated under the same conditions described in A without DNase I included in the pretreatment.

A**B**

situ nick translation and fixed tissue sections of 7.5 day embryos.

The advantage of using this approach is that the need for large numbers of embryos is greatly reduced. The second advantage is that slide preparations of single, fixed 7.5 day embryos are easily prepared and the DNase I sensitivity of cells from different tissues can be assessed in situ. In addition to this, tissues of embryonic and extraembryonic origin are all on the same slide, from the same embryo and therefore subjected to the same experimental conditions. Specific use of 7.5 day embryos allows one to 1) assess the DNase I sensitivity of relatively pure or uncontaminated embryonic and extraembryonic tissues and 2) to compare levels of DNase I sensitivity with the overall levels of DNA methylation reported for the same tissues of 7.5 day embryos by Chapman et al. (1984).

A standard in situ nick translation treatment using 4 ng/ml DNase I and 0.3 μM [^3H]-TTP was carried out at 14 C for 40 minutes. The radioactively labelled nucleotide was used so that levels of DNase I sensitivity could be quantitatively assessed. Nucleotide incorporation was visualized by autoradiography and the level of DNase I sensitivity for each tissue type was quantitated using mean net grain counts of nuclei. Sections of 7.5 day embryos in situ nick translated with and without DNase I are shown in Figure 19 A and B. Nuclei from embryo, extraembryonic ectoderm and ectoplacental cone, in situ nick translated with DNase I are shown in Figure 20 A, B and C, respectively. Nuclei from ectoplacental cone tissue, not treated with DNase I are shown in Figure 20 D. A denser grain distribution can be seen over embryo and extraembryonic ectoderm nuclei than with ectoplacental cone, with some of the dark staining

Figure 19. 7.5 day embryo section in situ nick translated with 4 ng/ml
DNAse I and $0.3 \mu\text{M}$ ^3H -TTP at 14 C for 40 minutes. Nucleotide
incorporation was visualized by autoradiography and sections
stained using hematoxylin-eosin. A. DNAse I treated.
B. Control treated. (40 X magnification)

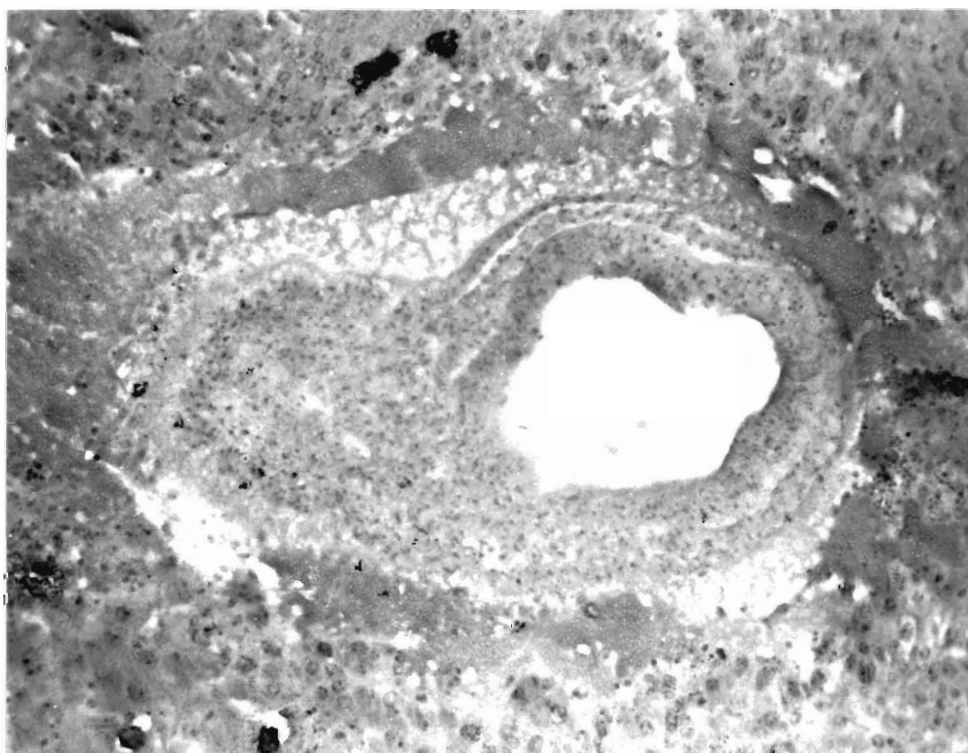
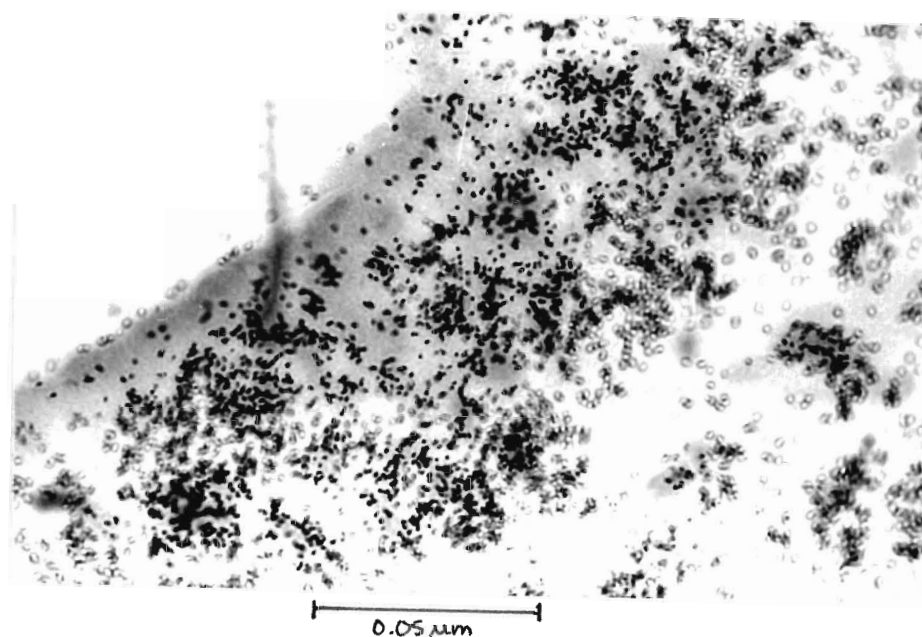
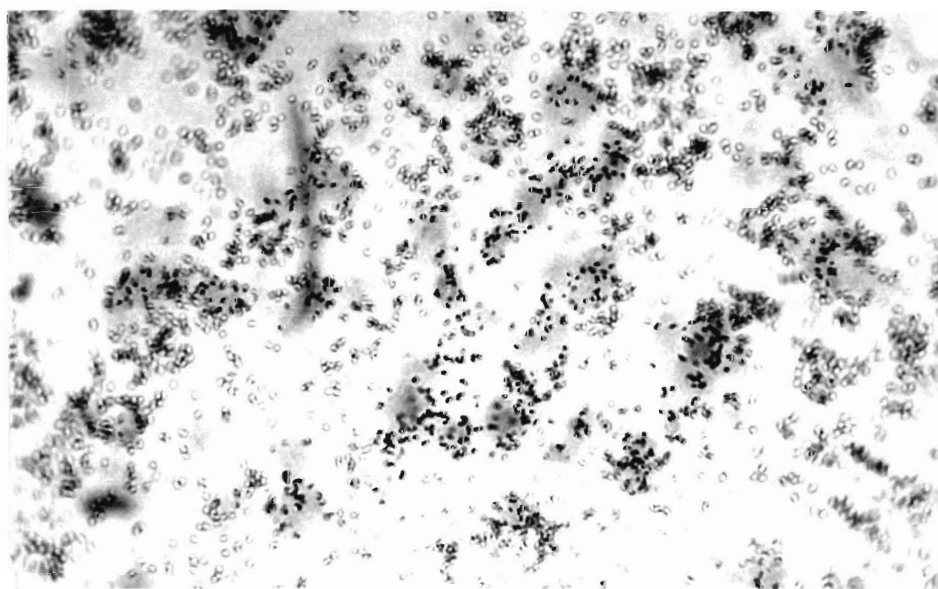
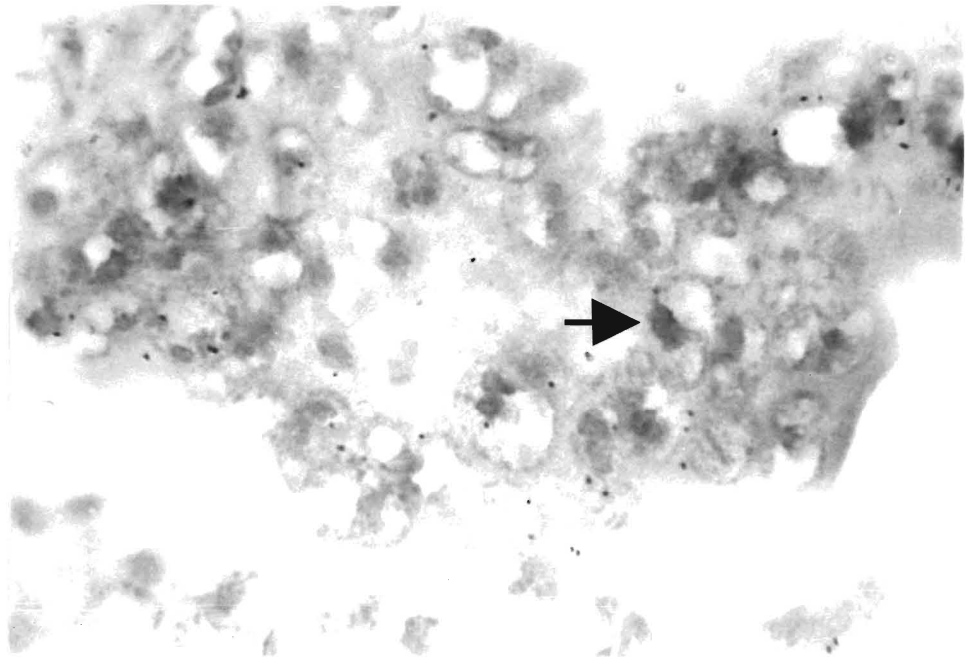
A**B**

Figure 20. Nuclei from A. embryo, B. extraembryonic ectoderm, C. ectoplacental cone 7.5 day embryo tissue sections in situ nick translated with 4 ng/ml DNase I and $0.3 \mu\text{M}$ $^3\text{[H]-TTP}$ for 4 minutes at 14 C. D. Control treated nuclei from ectoplacental cone tissue (see following page). Dark staining heterochromatic regions of nuclei are shown by arrow. (100 X magnification).

A**B****C**

D



0.05 μm

heterochromatic regions escaping labelling.

The results of in situ nick translated tissue sections obtained from 5 embryos are shown in Table 9. Twenty nuclei were analysed to achieve mean net grain count values per nucleus for each tissue examined. Significant differences in the level of labelling were tested using the Wilcoxon signed rank sum test and a P critical value of 0.05. Of the five embryos available for analysis, differences were detected in the DNase I sensitivity between emb, ex and epc in four of the embryos. It was generally observed that emb and ex were characterized by comparable levels of labelling while epc was found to be the least DNase I sensitive. In embryo 4, similar trends were observed with comparable mean net grain counts per nucleus for emb and ex and a lower value for epc. It is possible that the values obtained for this embryo were too variable to be statistically significant.

Microscopic examination of epc nuclei revealed large regions of darkly stained nuclear material, indicative of heterochromatin (Figure 20D). The heterochromatic regions were generally not labelled with silver grains. Since these cells were characterized by observable regions of heterochromatin, one would therefore predict this cell population to be less DNase I sensitive.

The general trends observed for overall levels of DNase I sensitivity did not correspond to the levels of DNA methylation reported in embryonic and extraembryonic tissues of 7.5 day mouse embryos by Chapman et al. (1984). Embryonic ectoderm, representative of the embryo proper, was

TABLE 9. OVERALL DIFFERENCES IN DNase I SENSITIVITY SIGNIFIED BY MEAN NET GRAIN COUNT OF NUCLEI ANALYSED FROM 7.5 DAY EMBRYONIC ECTODERM (EMB), EXTRAEMBRYONIC ECTODERM (EX) AND ECTOPLACENTAL CONE (EPC) TISSUE SECTIONS IN SITU NICK TRANSLATED USING 4 ng/ml DNase I, 0.3 μ M 3 H-TTP FOR 40 MINUTES AT 14°C.

Embryo	Mean Net Grain Count Per Nucleus			Statistical Significance
	EMB	EX	EPC	*(P=0.05)
Experiment 1				
1	37.2 ± 11.8	34.2 ± 19.8	14.9 ± 13.8	emb = ex emb > epc * ex > epc *
2	8.9 ± 4.5	7.6 ± 4.9	5.9 ± 5.9	emb = ex emb > ex * ex = epc
Experiment 2				
3	14.8 ± 6.8	14.3 ± 9.7	7.9 ± 6.1	emb = ex emb > epc * ex > epc *
	12.6 ± 5.9	21.0 ± 7.9	11.1 ± 8.5	ex > emb * emb = epc ex > epc *
4	7.3 ± 4.5	7.5 ± 4.4	4.2 ± 6.3	emb = epc = ex
5	16.5 ± 8.4	17.8 ± 8.6	7.5 ± 3.9	emb = ex emb > epc * ex > epc *
* Wilcoxon Signed Rank Sum Test P = 0.05				

characterized by a higher level of DNA methylation than ex and epc but was found to possess a relatively high level of DNase I sensitivity. Ex, known to be hypomethylated compared to emb, demonstrated comparable levels of DNase I sensitivity. Ectoplacental cone, also hypomethylated, was the least DNase I sensitive of all three tissue types.

These results indicated that in situ nick translation was useful for distinguishing differences in the level of DNase I sensitivity of cells from different tissue types. This is particularly useful when amounts of tissue are limiting. Differences were detected in the DNase I sensitivity between emb, ex and epc in 4 of the 5 embryos analysed. Since emb and ex were found to be more DNase I sensitive than epc, which is characterized by large regions of facultative heterochromatin, tissue-specific modification of DNA by chromatin structure may be implicated as a possible underlying mechanism controlling transcriptional activity in epc.

DISCUSSION

The initial aim of this study was to develop a means of looking at X-inactivation in the developing mouse embryo. Recently, the use of DNase I directed techniques have demonstrated differences in the DNase I sensitivities of euchromatic and heterochromatin regions of mitotic chromosomes. Kerem et al. (1983) reported that in situ nick translation could distinguish the active and inactive X chromosome of the gerbil on the basis of differing DNase I sensitivity, suggesting structural differences in chromatin between the two chromosomes. However, the recent controversy reported in the literature cast some doubt as to whether the inactive X chromosome in non-translocation type karyotypes could be distinguished by differential DNase I sensitivity (Kerem et al. 1983; Murer-Orlando and Peterson, 1985; Adolph and Hameister, 1985). Therefore, the primary goal of this study was to carry out a detailed evaluation of the potential of in situ nick translation for distinguishing differences in the DNase I sensitivity of active and inactive mouse and human chromosomes. At the same time the ability of in situ nick translation to distinguish constitutive heterochromatin was also assessed since this had also been the subject of some controversy. Finally the ability of DNase I to detect overall differences in chromatin structure was assessed by in situ techniques. The use of in situ nick translation did not distinguish the active X chromosome from the inactive X chromosome in either mouse or human tissues but regions of constitutive heterochromatin could be distinguished by their relative DNase I insensitivity. In situ nick

translation of 7.5 day mouse embryo tissue sections detected differing levels of DNase I sensitivity between different embryonic tissue with embryonic ectoderm and extraembryonic ectoderm possessing comparable levels of DNase I sensitivity while ectoplacental cone was significantly less DNase I sensitive.

IN SITU DNase I-DEPENDENT NICK TRANSLATION:

Since in situ nick translation is a relatively recently described procedure, it was important to establish that the specific labelling observed in interphase nuclei and mitotic chromosomes was actually DNase I dependent. Initial experiments using ³[H]-TTP monitored the effect of increasing DNase I treatment time and concentrations on the labelling of cell spreads. The positive correlation observed between increased DNase I treatment time and increased labelling of interphase nuclei suggested that labelling by in situ nick translation was indeed DNase I dependent. The use of ³[H]-TTP and autoradiography to visualize nucleotide incorporation was not without inter- and intra-experimental variability which was a main limitation of the technique.

The use of bio-16-dUTP along with immunocytochemical and enzymatic detection to visualize nucleotide incorporation improved the specificity of chromosomal labelling by in situ nick translation and reduced variability. The use of a biotinylated nucleotide as an alternative probe to radioisotopes is also a relatively recent and novel concept (Langer et al, 1981). Because of this, it was necessary to define in situ nick translation treatment times and conditions for visualization of nucleotide incorporation. The introduction of immunocytochemical treatment with

primary and secondary antibodies prior to enzymatic detection, based upon conditions defined by Murer-Orlando and Peterson, (1985), further enhanced the sensitivity of detection. When incorporation was visualized by SAV-HRP enzymatic detection alone, the intensity of staining was much less, indicating that the mechanism of antibody detection does increase sensitivity. The use of bio-16-dUTP in the in situ nick translation³ reaction, unlike [³H]-TTP, resulted in the labelling of all DNase I-treated cells without the limiting variability observed in labelling. In situ nick translation using bio-16-dUTP provided a reliable means of assessing the DNase I sensitivity of fixed mitotic cells.

ANALYSIS OF BANDING PATTERNS:

In situ nick translation of mouse mitotic cells using [³H]-TTP did not result in a clear banding pattern but a symmetrical labelling pattern was observed in similar positions on both chromatids. All chromosomes were labelled with the exception of the centromeric regions of some of the chromosomes. This type of banding pattern was consistently observed in other studies defining in situ nick translation conditions (Gazit et al., 1982; Kerem et al., 1983; Kerem et al., 1984, Murer-Orlando and Peterson, 1985).

In situ nick translation using bio-16-dUTP resulted in a more specific labelling of different areas of mouse and human chromosomes. Mouse chromosomes of adult and embryonic origin were generally characterized by minimal chromosomal banding with the centromeric regions of many

chromosomes again escaping labelling. Since centromeric regions are highly enriched with constitutive heterochromatin known to be condensed in structure and transcriptionally inert, their relative DNase I insensitivity provided good evidence that in situ nick translation is able to distinguish active and inactive regions of chromosomes. Human chromosomes were defined by a more extensive banding pattern, consisting of unstained and stained chromosome bands. The use of the biotinylated nucleotide in the in situ nick translation reaction has been reported, in a number of studies, to produce specific light and dark chromosome bands, termed D-bands (Kerem et al., 1983; Murer-Orlando and Petterson, 1985; Adolph and Hameister, 1985).

Recently, there has been much controversy in the literature as to whether the chromosomal banding pattern produced by in situ nick translation actually corresponds to transcriptionally active regions of chromatin. Kerem et al. (1984) and Murer-Orlando and Peterson (1985) both reported that in situ nick translation differentiates constitutive heterochromatic regions of chromosomes. Nick translation of the human Y chromosome, known to possess a large transcriptionally inactive constitutive heterochromatic segment, revealed this region to be DNase I insensitive. Adolph and Hameister (1985) on the other hand, contend that the heterochromatin of the Y chromosome was as equally stained as the euchromatic region. Instead, they reported a preferentially DNase I sensitive site located at the euchromatic-heterochromatic boundary. Their conclusions, however, are based upon compiled data, in which 4 out of 7 Y chromosomes showed this preferential DNase I sensitive boundary. Three chromosomes demonstrated a less intense labelling pattern of the

heterochromatic region. The question of whether in situ nick translation distinguishes constitutive heterochromatic regions was addressed in the present study by examining human chromosomes that are known to contain constitutive heterochromatin. Secondary constriction sites containing large segments of constitutive heterochromatin in human chromosomes 1, 9 and 16 were generally not labelled or less DNase I sensitive than other regions. In addition to this, the predominance of label found at the distal ends of both mouse and human chromosomes is consistent with the knowledge that transcriptionally active genes usually reside on the ends of chromosomes (Kuo and Sanders, 1977). The distinct labelling pattern produced of human chromosomes and the relative DNase I insensitivity of constitutive heterochromatin in both mouse and human chromosomes shown in this study suggested that in situ nick translation can distinguish regions of different transcriptional activity.

The variability reported in the literature can be attributed to a variety of mechanisms, many of which are technical in nature. The use of autoradiography is generally limited by the exposure of the silver grains in the photographic emulsion by decaying ³[H]-TTP. A more discrete distribution of labelling was observed in mouse chromosomes in situ nick translated using the biotinylated nucleotide than ³[H]-TTP. Detection of labelling by way of precipitate formation is a much more sensitive and direct as it is a staining process. The use of immunocytochemical treatment as well as enzymatic detection ensures that precipitate formation is truly representative of biotinylated DNA and not due to endogenous peroxidase activity found in contaminating cell cytoplasm (Manuelidis et

al., 1982). The extent of the chromosomal banding pattern produced was also very much dependent upon the the length of DNase I treatment. Murer-Orlando and Peterson (1985), and Adolph and Hameister (1985) both reported that the intensity of labelling achieved with bio-16-dUTP was relatively constant with a variety of DNase I concentrations and treatment time. It was observed in this study that although the presence of chromosomal banding patterns was probably not strongly influenced by DNase I treatment time in terms of the percentage of metaphases labelled, the intensity of precipitate formation was. Human chromosomes treated with DNase I for longer treatment times were more intensely labelled than those subjected to shorter treatment times and the quality of chromosomal banding became less distinct. There was less of a difference observed between the light and dark bands as the light bands had a tendency to become more stained. Because of extended DNase I treatments, DNase I insensitive regions could subsequently appear more DNase I sensitive. Production of discrete banding patterns was also greatly dependent upon chromosome quality. Chromosomes that were shorter and more condensed or contaminated by cytoplasmic debris were less likely to be banded than longer chromosomes. However, overstaining and overtreatment of chromosomes or poor chromosome morphology are limitations of any chromosomal staining technique and are not necessarily specific limitations of the in situ nick translation assay. It does however stress the importance of defining conditions that result in the most optimal or effective labelling of mitotic cells.

ANALYSIS OF X-CHROMOSOME LABELLING :

Kerem et al. (1983) initially reported that the inactive X chromosome of the gerbil could be distinguished from the active X chromosome by in situ nick translation. This would suggest that the chromatin conformation of the inactive X chromosome is altered from the active X chromosome. The inactive X chromosome appears to be highly condensed and is attached to the nuclear membrane throughout interphase (Barr and Bertram, 1949). Highly condensed mitotic chromosomes are transcriptionally inactive but regain their activity during S phase of the cell cycle, whereby the condensed chromatin regions become almost completely dispersed (Fakan and Nobis, 1978). Since the DNase I sensitivity conformation of potentially active genes is maintained during metaphase, detection of a DNase I-insensitive conformation by the inactive X chromosome would implicate chromatin conformation as a possible mechanism regulating inactivation. In this study, however, no differences in the DNase I sensitivity were detected between the active and inactive mouse or human X chromosomes by in situ nick translation. The use of the biotinylated nucleotide with the in situ nick translation reaction clearly demonstrated that all chromosomes were equally labelled. No single chromosome representative of the X chromosome in either mouse or human cells consistently escaped labelling. This observation has been supported by the results of two other studies, Murer-Orlando and Peterson (1985), and Adolph and Hameister (1985). In both studies, no differences were observed in the DNase I sensitivity between the active and inactive human or mouse X chromosomes.

A number of possible explanations have been put forward as to why a

difference was observed between the active and inactive X chromosome of the gerbil. Adolph and Hameister (1985) suggested that perhaps small quantitative differences in DNase I sensitivity could be more clearly observed using autoradiography. This, however, is not a likely explanation since the use of autoradiography to detect ³[H]-TTP incorporation was shown in this study to be limited by variability in labelling and discrete banding patterns were not very apparent in mouse chromosomes. A much more specific labelling pattern was observed in both mouse and human chromosomes using bio-16-dUTP. Constitutive heterochromatic regions of mouse and human chromosomes consistently escaped labelling when in situ nick translated ³with either [H]-TTP or bio-16-dUTP but the unlabelled regions were much more distinctive using the biotinylated nucleotide.

Riley et al. (1984) attributed the differential DNase I sensitivities of the gerbil X chromosomes to the selective fixing of chromosomes with methanol/glacial acetic acid. It was postulated that such treatment selectively fixes the chromatin of the inactive X chromosome by making it more DNase I resistant while at the same time inducing greater sensitivity of much of the active chromosome. Shapiro et al. (1978) presented evidence that acetic acid treatment denatures chromosomal DNA in situ. In the study reported by Riley et al., no differences were observed in the DNase I sensitivity of PGK sequences from either the active or inactive X chromosome in human cells, although a DNase I sensitive site was observed on the active X chromosome.

The effect of methanol/glacial acetic acid on the DNase I sensitivity of metaphase chromosomes was assessed in this study using in vitro nick

translation of unfixed mouse mitotic cells. Very few differences were observed in the labelling pattern of in vitro nick translated unfixed cells and in situ nick translated fixed mitotic cells. All chromosomes of in vitro nick translated cell spreads appeared to be labelled. The only property that was not as well maintained with in vitro nick translation was chromosome morphology. This is not surprising since the acetic acid component of the fixative is known to preserve chromosome morphology (Shapiro et al., 1978). It appears then that the denaturing effect of acetic acid treatment was not detrimental enough to cause any significantly detectable changes in the DNase I sensitivity of chromatin nor did it selectively fix the inactive mouse or human chromosome.

The differential DNase I sensitivity observed of the gerbil X chromosome could be due to an inherent property of the chromosome itself. The gerbil X chromosome is a composite X chromosome, consisting of two autosomal segments translocated onto the "original X" chromosome. Perhaps the alteration of the original X chromosome to an X-autosomal translocation chromosome has resulted in a modification of chromatin that is visualized by enhanced DNase I insensitivity. Some species of rodents possess a composite X chromosome that does not behave in a facultative manner. Instead, only part of the X chromosome behaves as facultative heterochromatin and the remainder behaves as constitutive heterochromatin (Lyon, 1972). If this were the situation in the gerbil, the findings of Kerem et al. (1983) could be accounted for since in situ nick translation is able to distinguish constitutive heterochromatin.

Another reason why the inactive X chromosome of either the mouse or

humans perhaps did not display differential DNase I sensitivity should include the possibility that in situ nick translation may not be able to detect facultative heterochromatin. Facultative heterochromatin is defined by Lyon (1972) as chromosome material which can assume either the euchromatic or heterochromatin state at different stages of the cell cycle, in different homologous chromosomes within a cell, in different cell types or at different stages of development. Constitutive heterochromatin on the other hand, is more condensed, remains transcriptionally inactive throughout the cell cycle, in almost all cells and at different developmental stages. Constitutive heterochromatin is also thought to be chemically distinct from the rest of the chromatin since it consists mainly of repetitive DNA sequences (cited in Lyon, 1972). In addition to this, its replication pattern and mode of condensation are thought to be different than those of facultative heterochromatin (cited in Lyon, 1972). It could be that facultative heterochromatin is not distinct enough in structure from euchromatin to be distinguished by DNase I but constitutive heterochromatin is.

One could also suggest that differences between euchromatin and heterochromatin in mitotic chromosomes are not distinct enough to be distinguished by in situ nick translation. The bulk of the chromatin that exists in interphase cells, in vivo, is made up of a 25-30 nm chromatin fiber that is condensed to give a DNA packing ratio of 40-50:1 (Finch and Klug, 1980). The additional folding of the chromatin fibers in metaphase chromosomes condenses the 30 nm fiber to such a degree that a compaction factor of 400-800 or more is achieved (Lewis and Laemmli, 1982). This

high degree of compaction found in metaphase chromosomes could limit the ability of in situ nick translation to distinguish between facultative heterochromatin and euchromatin but not between constitutive heterochromatin and euchromatin. During interphase, much of the chromatin is dispersed (Fakan and Nobis, 1978), while the inactive X chromosome, an example of facultative heterochromatin, becomes condensed as a Barr body (Barr and Bertram, 1949). It is possible that structural differences between euchromatin and facultative heterochromatin are more accentuated in interphase nuclei since the difference between condensed and extended chromatin is much more pronounced than in mitotic chromosomes. The latter explanation seems the most plausible since differences between facultative heterochromatin and euchromatin were detected in interphase nuclei of 7.5 day embryo sections by in situ nick translation, as will later be described.

OVERALL DNase I SENSITIVITY ASSESSMENT OF SPECIFIC EMBRYONIC TISSUES:

The use of in situ nick translation to distinguish differences in the DNase I sensitivities of different tissue types is a new application that has been developed in this study. One of the major issues considered in mammalian development is the concept of differential gene regulation and its role in differentiation. The results of Liskay and Evans (1980), Chapman et al. (1982) and Kratzer et al. (1983) collectively indicated that differences in DNA modification existed between the active and inactive X chromosome in adult and embryonic tissues but not in extraembryonic tissues. This would suggest that some X-chromosome

regulating mechanism exists that is specific for extraembryonic tissues in the mouse embryo. In order to better understand the underlying mechanism regulating X chromosome activity during embryonic development of the mouse, Chapman et al. (1984) asked whether overall differences in DNA modification by methylation could be detected in embryonic and extraembryonic cell lineages. The fact that extraembryonic tissues were less methylated than embryonic tissues suggested that gene regulation dependent upon DNA modification was different in the extraembryonic cell lineages. This difference could account for the lowered level of DNA modification observed of the inactive X chromosome from 14 day yolk sac tissues. Possible mechanisms regulating differential gene expression and perhaps X-inactivation in different embryonic cell lineages were further assessed in this study by quantitating overall levels of DNase I sensitivities of embryonic and extraembryonic tissues. Developmental regulation of chromatin structure has been reported for β and α -globin genes and the lysozyme gene in the chicken using DNase I sensitivity studies (Stalder et al., 1980; McGhee et al., 1981; Fritton et al., 1984). States of cell differentiation can also be characterized by differences in DNase I sensitivity. Generalized alterations in chromatin conformation were distinguished between teratocarcinoma stem cells and differentiated cells (Huebner et al., 1981). Murer-Orlando and Peterson (1985) also observed an increase in DNase I sensitivity of very early preimplantation mouse embryo chromosomes that was correlated with progressive developmental stages. From these results, chromatin structure may be considered as a mechanism regulating gene activity in different tissue types of the developing

embryo.

In situ nick translation of 7.5 day mouse embryo tissue sections revealed embryonic ectoderm and extraembryonic ectoderm to be more DNase I sensitive than ectoplacental cone. These general trends did not correspond to the levels of methylation reported in embryonic and extraembryonic tissues of 7.5 day embryos reported by Chapman et al. (1984). Embryonic ectoderm, highly methylated in the embryo, was found to be the most DNase I sensitive. Extraembryonic ectoderm, an extraembryonic tissue that is undermethylated in the 7.5 day embryo, was found to be comparably DNase I sensitive. Ectoplacental cone, also undermethylated in the embryo, was found to be the least DNase I sensitive of the two extraembryonic tissues. These results suggest that in situ nick translation is able to detect differences in the DNase I sensitivity of cells from different tissues particularly when amounts of tissue are limiting.

The differences in DNase I sensitivity observed between epc, emb and ex suggest that tissue-specific modification of DNA by chromatin structure may be involved as a mechanism controlling transcriptional activity. The lower levels of DNA methylation observed in the 7.5 day embryo by Chapman et al. (1984) indicate that DNA modification by methylation may not be the primary mechanism involved in gene regulation. DNase I sensitivity of the embryonic tissues suggest that they are potentially active at the transcriptional level. The high levels of DNA methylation observed in emb tissues and the enhanced DNase I sensitivity reported in embryo derived tissues suggest that DNA methylation could be involved in a more secondary way as a regulating mechanism than chromatin structure. The enhanced DNase

I sensitivity of the extraembryonic ectoderm correlated with hypomethylation followed the correlation generally observed for methylation and DNase I sensitivity. Direct conclusions from this correlation are difficult. Enhanced DNase I sensitivity again suggested that extraembryonic ectoderm in the 7.5 day mouse embryo is transcriptionally active or at least potentially active. Low levels of methylation suggest that DNA methylation is probably not a primary mechanism regulating gene activity in this tissue. The lower level of DNase I sensitivity characterizing ectoplacental cone, implies that the chromatin configuration of epc is altered from that of emb and ex. Reported hypomethylation of this tissue also suggests that DNA methylation is probably not a primary regulator of gene activity. The differences observed in the DNase I sensitivity of ex and epc also implies that chromatin structure as a mechanisms regulating tissue-specific activity cannot be globally applied to all extraembryonic tissues. Whether epc is less transcriptionally active than ex or emb is not known. One could ask whether levels of RNA synthesis are lower in epc than in the other two tissues. The results do suggest, however, that tissue specific mechanisms such as chromatin structure could be involved in controlling gene activity in different cell lineages of the developing mouse embryo.

POSSIBLE MECHANISM REGULATING X-INACTIVATION:

The next obvious question is how do general mechanisms regulating transcriptional activity of specific tissues apply to X-inactivation or do they? It was initially proposed by Mohandas et al. (1981), that

X-chromosome inactivation involves DNA methylation. Three hypotheses have been proposed as to how DNA methylation could possibly regulate the facultative heterochromatinization of the X chromosome (Miller, 1982).

X-inactivation could involve the methylation of a small number of sites or genes on the inactive X-chromosome. This requires an "inactivation centre" that becomes methylated on the X-chromosome to be inactivated. The second hypothesis suggests that all genes on the inactive X chromosome are methylated, leading to the inactivation of the whole chromosome and the third hypothesis proposes that X-inactivation is the result of every methylation site on the X-chromosome being methylated.

The use of antibodies to 5-methylcytidine (Miller et al., 1982) and 5-azaC (Wolf and Migeon, 1982) demonstrated that the active and inactive X chromosomes are not characterized by significantly different levels of methylation nor are sequences in the X chromosome. The HPRT gene does exhibit differing levels of DNA methylation on the active and inactive X chromosomes (Mohandas et al., 1981; Wolf et al., 1984; Yen et al., 1984). The gene was found to be undermethylated on the active X chromosome but different and highly variable patterns of DNA methylation were observed on the inactive X chromosome, mainly of sequences found 5' to the HPRT gene. Treatment of cells with 5-azaC has resulted in the reactivation of HPRT, G6PD and PGK genes albeit at a reduced level (cited in Miller, 1985). These results collectively suggest that the first hypothesis is the more realistic of the three. DNA methylation appears to be more involved in the regulation of specific genes than in the overall regulation of the X chromosome.

The condensed nature of the X chromosome during a large part of the cell cycle suggests that chromatin structure may be a more applicable mechanism regulating X chromosome activity. Referring to the lower levels of methylation reported in the ectoplacental cone of the 7.5 day mouse embryo and the relative DNase I insensitivity observed in this study could imply that chromatin structure is involved as a regulating mechanism. Since tissue-specific levels of DNA methylation have been reported in a variety of studies and have been proposed to play a role as regulating mechanisms of gene activity, this could also be applied in terms of tissue-specific chromatin configurations. The lowered level of DNA modification of the inactive X chromosome in epc could imply that chromatin structure is involved in the regulation of X-chromosome activity. Methylation could then be involved as a secondary mechanism, controlling the transcriptional activity of certain genes by altering the structure of DNA and consequently that of chromatin. This could cause the DNA of the inactive X chromosome to interact differently with histones, non-histone proteins or regulatory proteins. The use of in situ techniques and specific probes could be then used to detect differences in the DNase I sensitivity of repetitive sequences on the X chromosome and would provide further insight as to whether chromatin conformation influences X chromosome activity.

The precise mechanisms regulating X chromosome activity are still not clear but whatever the mechanisms, they must account for the many events associated with X-chromosome activity. The correlation of chromatin structure with potential gene activity, DNA modification by methylation,

and its influence on the interaction of DNA-binding proteins, histone and non-histone proteins make it an attractive candidate as a potential mechanism regulating X-inactivation.

CONCLUDING REMARKS: "THE FUTURE OF IN SITU NICK TRANSLATION"

In situ nick translation was effective in distinguishing known regions of transcriptionally active and inactive regions of chromosomes, it did not however, distinguish the active and inactive X chromosomes of mouse and human tissues. Only constitutive heterochromatin was distinguished from euchromatin. In situ nick translation also proved to be useful in distinguishing differences in the level of DNase I sensitivity of cells from different tissue types. It allows one to assess gross differences in chromatin structure at a general level. The advantages of using in situ nick translation are that the DNase I sensitivity of a tissue can be detected in situ as opposed to in vitro and can be used when amounts of tissue are limiting. The limitation of this assay is that it is only able to distinguish regions of differing DNase I sensitivities at a gross level, making detection of single copies of genes virtually impossible. In order to further assess levels of DNase I sensitivity of different tissue types at the molecular level, probes for specific genes or repetitive sequences would have to be used. This would allow one to look at specific genes that are actively transcribed at different stages of development in different cell lineages of the mouse embryo. One could then ask how or if chromatin structure is involved in the differential regulation of such genes, is it a tissue specific mechanism and how is it involved in

differentiation to give a better understanding of transcriptional control in the developing mouse embryo.

SUMMARY: THE MAIN FINDINGS OF THIS STUDY

1. In situ DNase I dependent- nick translation distinguished regions of mitotic chromosomes with differing transcriptional activities.
2. Facultative heterochromatin of the inactive X-chromosome could not be distinguished by in situ nick translation.
3. Constitutive heterochromatin of mitotic chromosomes could be distinguished by the same technique.
4. Differences in overall DNase I sensitivity can be detected between tissue types by in situ techniques, particularly when amounts of tissue are limiting.
5. Differences were observed in the DNase I sensitivity between embryonic ectoderm and extraembryonic ectoderm, and ectoplacental cone.

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APPENDIX 1: PREPARATION OF MEDIA AND REAGENTS

COMPLETE ALPHA MEM

To make up 100 ml:

88 ml alpha base (Gibco MEM alpha with glutamine, without nucleosides)

1 ml streptomycin (stock 5.0 mg/ml)

1 ml penicillin (stock 5.97 mg/ml)

5 ml fetal calf serum (Flow Labs., heat inactivated)

5 ml newborn calf serum (Flow Labs., heat inactivated)

Sterilized medium with Millipore filter, 0.45 μ m pore size.

PHOSPHATE BUFFERED SALINE (PBS)

To make up 1 litre:

100 mg CaCl₂

200 mg KCl

200 mg KH₂PO₄

59.2 mg MgSO₄

8.0 g NaCl

1.15 g Na₂HPO₄

+2 +2
For Mg²⁺ -, Ca²⁺ - free PBS, excluded CaCl₂ and MgSO₄.

Sterilized with 0.22 μ m pore size Millipore filter

ACID TYRODES SOLUTION

To make up 100 ml:

0.8 g NaCl

0.02 g KCl

5.6 mg NaH_2PO_4

0.1 g glucose

0.1 g NaHCO_3

0.4 g polyvinyl pyrrolidone

The pH of the solution was adjusted to 2.5 and sterilized using a 0.22 μm pore size Millipore filter.

2.5% PANCREATIN-0.5 % TRYPSIN SOLUTION

To make up in 5 ml of Ca^{+2} -, Mg^{+2} -free Acid Tyrodes solution:

25 mg trypsin

125 mg pancreatin

The solution is well mixed and filtered using Whatman paper filter No. 4 at 4 C and then refiltered using a 0.45 μm pore size Millipore filter and kept at 4 C.

HEMATOXYLIN-EOSIN STAINING SERIES

hematoxylin	1 min
H ₂ O rinse	
2	
30 % ethanol	1 min
50 % ethanol	1 min
70 % ethanol	1 min
eosin	15 seconds
70 % ethanol	1 min
80 % ethanol	1 min
90 % ethanol	1 min
95 % ethanol	1 min
absolute ethanol	2 min
absolute ethanol/ xylene	2 min
xylene	5 min
xylene	5 min

EHRlich'S ALUM HEMATOXYLIN

6 g hematoxylin
 600 ml absolute ethanol
 60 ml acetic acid
 600 ml glycerol
 600 ml distilled water
 200 g aluminum ammonium sulfate

The stain is allowed to age for 6 weeks, filtered and diluted 1:2 with distilled H₂O before use.

EOSIN

1 g alcoholic eosin in 100 ml 80 % ethanol

1 g yellowish eosin in 100 ml H O

1 g bluish eosin in 100 ml 30 % ethanol

GIEMSA STOCK

0.8 g Giemsa

50 ml methanol

50 ml glycerol

GIEMSA STAIN

5 ml stock Giemsa

45 ml phosphate buffer (0.07 M Na HPO₂ , 0.07 M KH PO₂), pH 6.8

Filter.

PHOSPHATE BUFFER (pH 6.8)

To make up 250 ml each of:

0.07 M Na HPO₂ 4

0.07 M KH PO₂ 4

2.37 g Na HPO₂ 4 in 250 ml distilled H O 2

2.7 g KH PO₂ 4 in 250 ml distilled H O 2

Add equal amounts of each.

2 % ACETO-ORCEIN STAIN

2 g orcein in 45 ml hot glacial acetic acid

Boil solution gently with stirring for 3-5 min. Cool and add 55 ml distilled H₂O to give a 2 % solution in 45 % acetic acid.

Filter before use.

PANCREATIC DNase I

1 mg/ml DNase I in:

150 mM NaCl

50 % glycerol

Volume adjusted with DEPC.H₂O and stored at -20 °C.

RNase A

10 mg/ml pancreatic RNase A in :

10 mM Tris-HCl, pH 7.5

15 mM NaCl

Adjust volume with ultra-pure H₂O, heat to 100 °C for 15 minutes and cool to room temperature. Store at -20 °C.

PROTEINASE K

3 mg/ml Proteinase K in:

5 % SDS

200 mM EDTA, pH 8.0

100 mM Tris-HCl, pH 7.5

Volume is adjusted with ultra-pure H₂O and stored at -20 °C.

DEPC.H₂O (DIETHYL PYROCARBONATE)
2

5 drops DEPC in 500 ml double distilled H₂O
2

Sterilize by autoclaving.

In situ NICK TRANSLATION BUFFER WORKING SOLUTION:

50 mM Tris-HCl, pH 7.9

5mM MgCl₂

10 mM 2-mercaptoethanol

50 µg/ml bovine serum albumin

For in situ nick translation add just before using:

4 µM dCTP, dATP, dGTP
3

0.3 µM [³H]-TTP

4 ng/ml DNase I

10 U/ml DNA polymerase I

For in vitro nick translation of nuclei:

4 µM dCTP, dATP, dGTP

3 µM dTTP
32

1 µM P-dTTP

DNase I-various concentrations

10 U/ml DNA polymerase I

In vitro NICK TRANSLATION BUFFER A

10 mM Tris-HCl, pH 7.5

10 mM NaCl

3 mM MgCl

₂

Containing NP-40 and TX-100 add:

0.5 % Nonidet P-40

0.5 % Triton X-100

In vitro NICK TRANSLATION BUFFER B

10 mM Tris-HCl, pH 7.5

5 mM MgCl

₂

5 mM 2-mercaptoethanol

Just before use add:

4 uM dATP, dCTP, dGTP

₃

50 μ Ci [H]-TTP

35 U DNA polymerase I

SPUN COLUMNS

1. Pack Sephadex G-50 in STE, pH 8.0, into a disposable 1 ml tuberculin syringe plugged with sterile glass wool.
2. Spin column with table top centrifuge at 1700 rpm for 5 min.
3. Rinse column two times with STE, centrifuging as previously described between each rinse.
4. Before adding radioactively labelled, nick translated DNA, rinse column once more with STE and centrifuge.

STE BUFFER

10 mM Tris-HCl, pH 7.6

100 mM NaCl

1 mM EDTA, pH 8.0

Volume adjusted with distilled H₂O and autoclaved.